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UNIVERSITY OF ALBERTA

INTERACTIONS OF AMPHIPHILIC ALKALINE PHOSPHATASE

BY

TRACY LEE KIHN

(C)

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

Fall 1991

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Interactions of amphiphilic alkaline phosphatase" submitted by Tracy Lee Kihn in partial fulfillment of the requirements for the degree of Master of Science in Experimental Pathology.

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Chairman of oral exam

DATE: Aug. 19, 1991

This thesis is dedicated to my husband Ron.

Abstract

Alkaline phosphatase in the plasma membrane of cells from human liver or placenta is anchored by glycosyl-phosphatidylinositol. When solubilized from whole cells or isolated plasma membranes with detergent or with butanol extraction at high pH, it retains this anchor, and the enzyme is amphiphilic. When phospholipases are used to release the enzyme, the anchor is cleaved off and a hydrophilic form of the enzyme results. The amphiphilic enzyme from liver is more sensitive to heat, is activated less by various phospholipids and albumin, and has a greater affinity for 3 monoclonal antibodies than the hydrophilic form. They are similar in substrate K_m values, and sensitivity to inhibitors and sodium dodecyl sulfate inactivation. Amphiphilic forms but not hydrophilic forms of alkaline phosphatase were able to incorporate into liposomes and adsorb to octyl-Sepharose. Hydrophobic proteins inhibited incorporation into liposomes. An intact anchor also allowed incorporation into red cell ghosts. Soluble amphiphilic alkaline phosphatase was cleaved to a hydrophilic form by phosphatidylinositol phospholipase C from Bacillus cereus, and when the alkaline phosphatase was anchored to a membrane, this cleavage caused release of the enzyme. The rate of release was dependent on the nature of the membrane environment, but no difference was found in the rate of cleavage of liver or placental enzyme. A monoclonal antibody to liver alkaline phosphatase inhibited both its

incorporation into liposomes and its release. Liposome-incorporated and soluble amphiphilic alkaline phosphatases had similar substrate K_m values, and sensitivity to inhibitors, heat, sodium dodecyl sulfate, phospholipids, and albumin.

In the sera of patients with cholestatic liver disease a high M_r form of alkaline phosphatase is often seen. The high M_r enzyme does not enter the gel in either gradient or standard pore polyacrylamide gel electrophoresis, unless solubilized by detergent or butanol, and then has the migration of the amphiphilic enzyme solubilized from plasma membranes of human liver. Both high M_r and plasma membrane forms of the solubilized enzyme incorporated into liposomes and adsorbed to octyl-Sepharose. The two forms also had similar activation by phospholipids and albumin, substrate K_m values, and sensitivity to heat, sodium dodecyl sulfate, and inhibitors. Alkaline phosphatase was released from high M_r particles by phosphatidylinositol phospholipase C, as was another liver plasma membrane enzyme 5'-nucleotidase. Phospholipase D did not release the enzyme. The form of alkaline phosphatase seen in cholestatic liver disease is most likely fragments of the liver plasma membrane that contains amphiphilic alkaline phosphatase.

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Abbreviations

AchE	Acetylcholinesterase
ALP	Alkaline phosphatase
AMP	2-amino-2-methyl-1-propanol
EAE	2-(ethylamino)ethanol
HDL	High density lipoprotein
LUV	Large unilamellar vesicle
LDL	Low density lipoprotein
mAb	Monoclonal antibody
MES	2-[N-Morpholino]ethanesulfonic acid
MLV	Multilamellar vesicle
5'-NT	5'-Nucleotidase
PAGE	Polyacrylamide gel electrophoresis
PI	Phosphatidylinositol
PI-PLC	Phosphatidylinositol phospholipase C
PI-PLD	Phosphatidylinositol phospholipase D
PMSF	Phenylmethylsulfonyl fluoride
pNPP	p-nitrophenylphosphate
SDS	Sodium dodecyl sulfate
SUV	Small unilamellar vesicle
VSG	Variant surface glycoprotein

Chapter 1

Review Of The Literature

A. Alkaline Phosphatase

Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1.) is a dimeric zinc metalloenzyme, that has an optimum pH of >10 *in vitro* [1,2,3]. It is a non-specific phosphomonoesterase that catalyzes both phosphohydrolysis and transphosphorylation [Fig 1.1]. The phosphoryl enzyme intermediate is formed at a serine residue at the active site [3,5]. Alkaline phosphatase (ALP) is a glycoprotein that has an absolute requirement for Zn^{2+}, and can be activated by Mg^{2+}. The enzyme is usually made up of two identical subunits [5]. It is widely distributed in nature, and is found in everything from yeasts and bacteria to most mammalian species, but has not been discovered in plants [5]. Mammalian ALP is located mainly at cell surfaces, and is present in most tissues and fluids [5].

To date, four different isoenzymes of ALP, each encoded for by a different gene, have been identified. These are placental, intestinal, germ cell, and liver/bone/kidney (tissue non-specific) ALP [6,7,8]. The liver, bone and kidney isoforms are encoded by the same gene, and are different due to post-translational modifications [6,8]. The gene coding for tissue non-specific isoenzyme is located near the end of the short arm of chromosome 1 (p36.1-p34) [9]. Placental, germ cell and intestinal genes are all

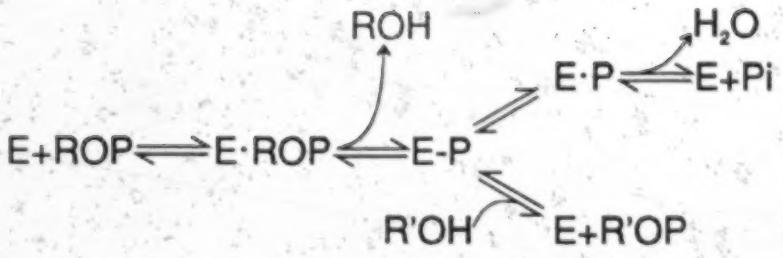

Fig. 1.1 Phosphohydrolysis and transphosphorylation reactions catalyzed by alkaline phosphatase [4]. The added nucleophile (R'OH) can react directly with the phosphorylated enzyme (E-P), while hydrolysis is only possible after isomerization to E·P.

located near the end of the long arm of chromosome 2 (q34-q37), and show considerable sequence similarity [10]. Placenta and germ cell (sometimes called placental-like ALP) have a sequence identity of 98%, and both have identity with the intestinal gene of about 87%. The tissue non-specific enzyme is only about 50-60% the same as the others [8]. There are fetal and adult intestinal forms of ALP, and these may be separate gene products [8,11]. The placental form is very polymorphic, and 3 common and >18 rare alleles have been identified [12]. Germ cell ALP is also polymorphic, but no allelic variants have been found for the other forms of ALP [8]. It is thought that the different ALP genes arose from one ancestral gene via gene duplication [8].

The highest concentration of tissue non-specific ALP in liver is found on the plasma membrane adjacent to the bile canaliculi, in bone on hypertrophic cartilage cells, osteoblasts and osteocytes, and in kidney on the brush border of the proximal tubule epithelial cells [13]. Intestinal isoenzyme is located mostly on the surface of the intestinal epithelial cell microvilli, and placental ALP on the surface of the trophoblastic syncytium [13]. Germ cell ALP is seen in testis and thymus [8]. The ALP in serum originates primarily from liver and bone, but intestinal, placental and kidney sources have also been identified [1,2]. Intestinal ALP can contribute to the serum ALP in about 25% of individuals, and is mostly seen in blood groups O and B and if secretor positive [6].

Techniques to differentiate the isoenzymes, and sometimes the isoforms, of ALP include thermal denaturation, chemical inhibition (due to urea, homoarginine, phenylalanine, levamisole, etc), chromatofocusing, a variety of types of electrophoresis, and immunological tests [3,6,8]. For example, the placental and germ cell isoenzymes are stable at 65°C for 1 h, but the other isoenzymes are not [8,14]. The bone isoform is more heat labile than the liver isoform [15]. Bone isoform of the tissue non-specific isoenzyme is the most susceptible to inactivation by urea, then liver isoform, with the intestinal and placental isoenzymes being quite resistant [1].

ALP is used in the routine diagnosis and observation of bone and hepatobiliary disorders, and is also used as a tumor marker [1]. Children normally have an increased serum ALP [1,17]. Serum ALP is also increased during pregnancy [12,18]. In children under ten years transient hyperphosphatasemia may be seen. They have an ALP many times that of the adult upper limit, but no evidence of liver or bone disease [1,19]. There is an increased serum ALP in bone diseases including Paget's disease, osteomalacia, metastatic carcinoma of the bone, and vitamin D deficient rickets [1,20]. In hepatobiliary disease there is an increased serum ALP, and a particulate form of ALP is also seen (see section B). A decreased leukocyte ALP is seen in chronic myeloid leukemia [5]. There is an increased

intestinal ALP in shock [20]. Bone or intestinal ALP can be elevated from hemodialysis [20].

Hypophosphatasia is an inborn error of metabolism which results in a decrease of tissue non-specific ALP in all tissues and serum [8,21,22], and a large amount of phosphoethanolamine and inorganic pyrophosphate are excreted in the urine [21,23]. The other isoenzymes are unaffected [24]. Defective osteogenesis is seen due to decreased bone mineralization, and dental and skeletal malformations occur [21,22]. There is a range of severity seen, with different forms resulting from a different genetic basis [7].

Tumor associated ALP includes placental, germ cell, and fetal intestinal isoenzymes [1,8]. The placental isoenzyme was first discovered in lung cancer and is seen in patients with a variety of carcinomas of the primary organs, premalignant conditions, and even at low levels in healthy people [25]. Germ cell isoenzyme is also found in carcinomas, particularly in seminomas [26]. The fetal intestinal isoenzyme may appear in patients with hepatoma or tumors of the GI tract [27].

The function(s) of ALP is unknown. ALP concentration is highest at the cell suface of tissues important for the transport of nutrients, and this location may be due to its function [5,13]. For example, intestinal ALP may be involved in the transport of inorganic phosphate [8]. Bone ALP is known to be involved in the mineralization of the bone, because of the effects seen in hypophosphatasia [8].

B. High Molecular Weight Alkaline Phosphatase

A high M_r form of ALP is found in serum in metastatic, infiltrative or cholestatic liver disease [28-33], occurring in 90% of patients with cholestasis [34]. The greatest elevations are seen in this latter condition, although the usefulness of identifying high M_r ALP has been questioned, since the total ALP is also invariably elevated in these patients [34]. Rather, better diagnostic information may be obtained in cases of suspected hepatic metastases, even though there is a high number of false positives [35]. High M_r ALP is sometimes seen in healthy adults but at much lower levels than the pathological conditions referred to [31,36]. As a result of cholestasis there is a marked increase in the rate of synthesis of ALP in liver, possibly mediated by cyclic adenosine monophosphate and activated by bile salts, steroids or a humoral factor in bile [34]. In bile duct-ligated rats this increased synthesis of ALP is a result of enhanced translation of mRNA rather than increased transcription [30]. A portion of liver ALP that enters the plasma as a result of cholestasis is complexed with other enzymes including 5'-nucleotidase (5'-NT), γ-glutamyl transpeptidase, leucine aminopeptidase, leucyl-naphthylamidase, $(Na^+ + K^+)$-Mg^{2+}-adenosine triphosphatase and nucleotide pyrophosphatase [33], and the result is a complex with an M_r >1 000 000 [31], referred to as high M_r ALP [32,36,37], particulate enzyme [29,31], high molecular mass

band [31,32], koinozyme [28,31], fast liver [33,38], α_1-isoenzyme [33], or biliary ALP [29].

Hepatocyte synthesized ALP may be initially incorporated into the sinusoidal membrane and then migrate to the canalicular membrane [34,39,Fig 1.2 & 1.3]. In cholestasis the increased pressure on the latter membrane may impair this movement and cause an accumulation of ALP in the sinusoidal membrane [39]. Enhanced solubilization of the enzyme would lead to increased plasma levels, and enhanced membrane fragmentation could lead to accumulations of plasma membranes in plasma [28-34,39-41]. Both of these processes could be promoted by the increased amounts of bile salts [37,39]. ALP, normally present as an integral but ecto plasma membrane protein, could then appear in plasma as a high M_r form that resembles plasma membranes under the electron microscope and in density [28,42]. Other evidence to support this hypothesis has been summarized by Sturk and Sanders: high M_r particles contain only enzymes that are found on the plasma membrane; high M_r complexes and liver plasma membranes are similar with respect to the specific activity of several enzymes; and spontaneous shedding of plasma membrane fragments has been shown to occur *in vitro* from human liver and *in vivo* and *in vitro* from various human cells [42].

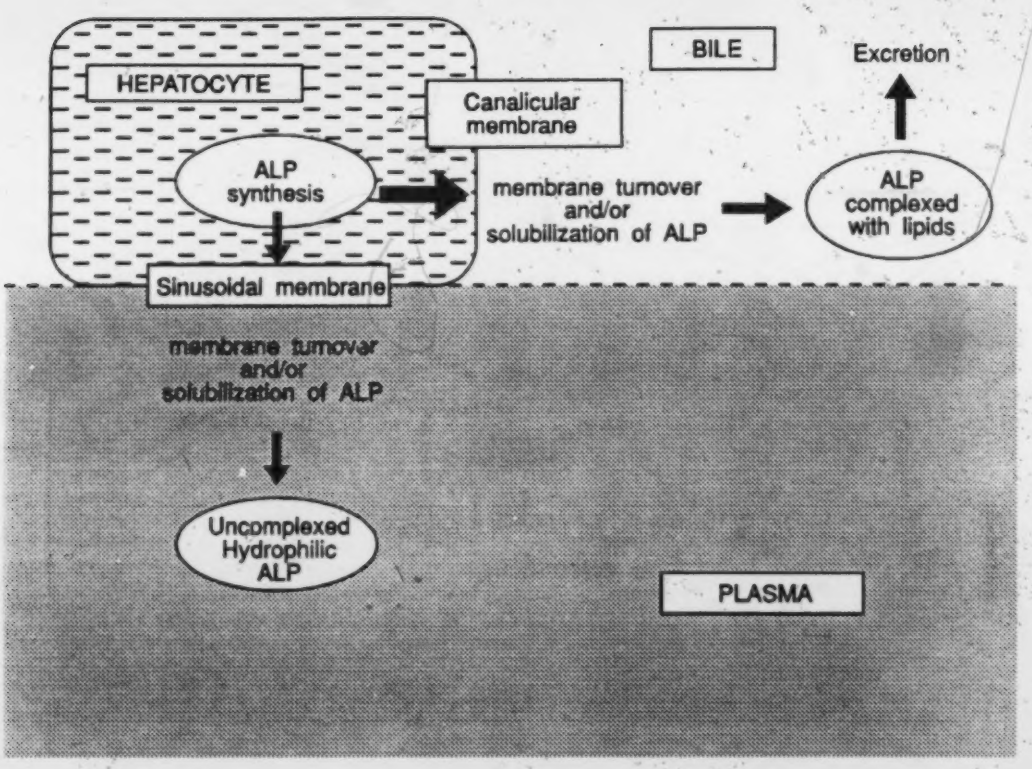

Fig. 1.2 Probable normal pathways of production of hepatic alkaline phosphatase. After synthesis in the hepatocytes most of the enzyme passes to the canalicular membrane and thence into the bile, either as a result of membrane turnover or the solubilizing action of bile salts. Non-covalent complexes with lipids are formed during or after this process, and the complexes are excreted with the bile. Some alkaline phosphatase passes to the sinusoidal membrane and into the plasma in an uncomplexed form and is cleared by tissue macrophages.

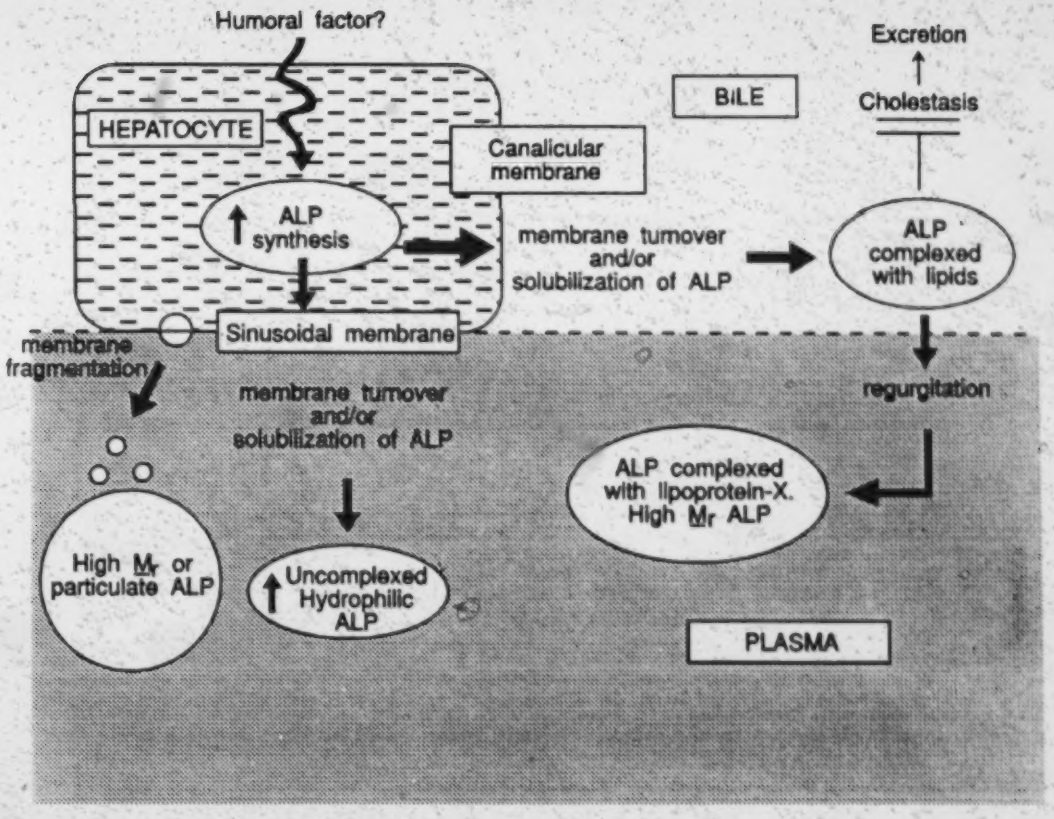

Fig. 1.3 Probable changes in metabolism of hepatic alkaline phosphatase in cholestasis. Alkaline phosphatase synthesis in hepatocytes is increased, and as a result of redistribution from canalicular to sinusoidal membranes, more enzyme passes directly to plasma, where the activity of the uncomplexed enzyme is correspondingly increased. Complexes of alkaline phosphatase formed in the bile are regurgitated into plasma where they can be detected as high molecular-mass complexes with lipoprotein-X. Additional high molecular-mass forms of alkaline phosphatase consisting of membrane fragments may also occur as a result of hepatocyte damage or destruction.

A second explanation of the nature of particulate ALP is that cellular ALP is solubilized by bile salts, and because of its hydrophobicity becomes complexed to lipoprotein-X [30,31,33,34,43], an association of phospholipids, cholesterol, apolipoprotein C, albumin, triglycerides and bile salts [33]. Lipoprotein-X is formed when the increased lipoproteins in plasma, as a result of the regurgitation of bile, react with serum components [44]. However, lipoprotein-X can also form in other disorders, whereas high M_r ALP is only found in cholestasis, so it is a more specific indicator of the disease [44].

It has been suggested that both forms of particulate ALP can exist simultaneously but are difficult to distinguish [31,33,43].

C. Anchoring by glycosyl-phosphatidylinositol

Alkaline phosphatase and many other proteins are anchored to the cell by covalent attachment of the C-terminal amino acid to glycan-phosphatidylinositol (PI) [for reviews see Ref. 45-56]. The number of proteins found to have this method of anchoring is growing (see Appendix A), and includes hydrolytic enzymes, cell adhesion molecules, parasitic coat proteins, and many proteins whose functions are unknown. The anchor was first identified because some proteins were released from cells with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) [48, see section D]. The detailed structure of the anchor has

been worked out for variant surface glycoprotein (VSG) of Trypanosomes, rat brain Thy-1, and human erythrocyte acetylcholinesterase (AChE) [46]. A common core structure for the anchor consists of PI-glucosamine-mannose$_3$-phosphoethanolamine [45,46,48,Fig 1.4]. The ethanolamine has an amide linkage to the α-carboxyl group of the C-terminal amino acid of the protein [48]. It is unusual to find non-N-acetylated glucosamine in mammalian carbohydrates, yet this component is common to all the anchors studied so far [46]. Outside of the common core structure, there are many variations for the anchor structure. The core mannosyl residues can be modified, the inositol can have an extra fatty acid attached, and the fatty acid groups that are linked to glycerol can vary [45].

An N-terminal sequence that directs the protein to the endoplasmic reticulum, and a C-terminal sequence of 20-30 amino acids that are mostly hydrophobic, are predicted by the cDNA of PI-anchored proteins, but the C-terminal portion is absent in the completed proteins, probably due to removal when the anchor is attached [57]. The addition of the PI anchor probably occurs in the endoplasmic reticulum because there is only a short period of time between the formation of the peptide and the addition of the anchor [58]. Also, the anchor is still attached when transport to the golgi from the endoplasmic reticulum is blocked [59]. The short time period also suggests that the anchor is preconstructed [58]. The same enzyme may catalyze the removal of the

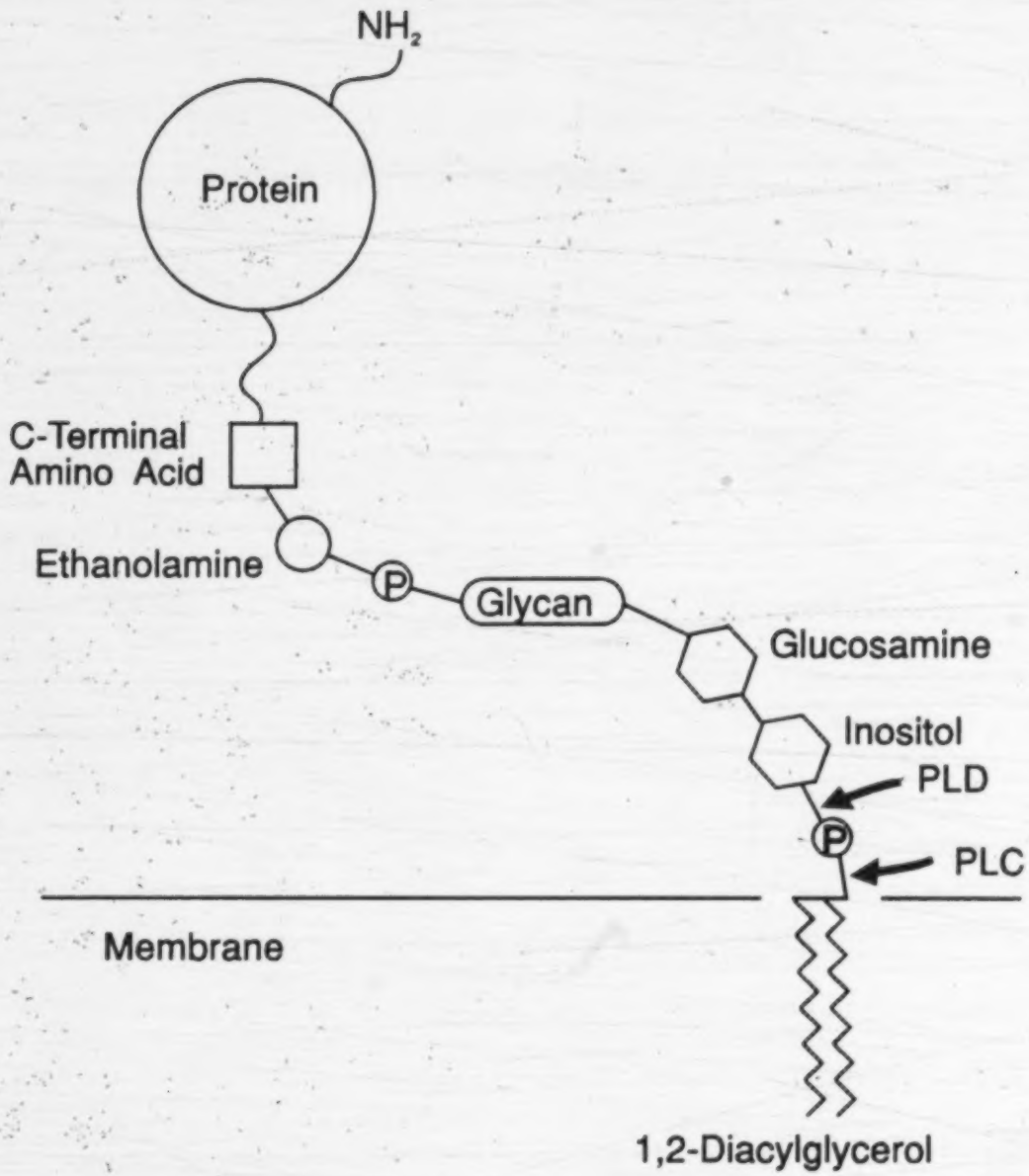

Fig. 1.4 Generalized structure of the PI anchor [50,72].

C-terminal peptides and the addition of the anchor, possibly a transpeptidase or a transamidase [46]. The function of the C-terminal peptide may be to anchor the protein to the endoplasmic reticulum until this transfer takes place [45]. There is no sequence homology found in the C-terminal end of the proteins which could be used as a signal for PI attachment [47]. The only similarity between these sequences is their degree of hydrophobicity, and unlike transmembrane domains they often include several charged or hydrophilic residues [60,61]. The sequence can be shortened and still work, but there is a minimum length after which attachment of the anchor no longer occurs [60,61]. Part of the signal for attachment of the PI anchor also comes from the first 20 residues after the cleaved C-terminal [62].

Some PI-anchored proteins have also been found in a peptide anchored or secreted form, probably due to differential splicing of their mRNA [46].

Most PI-anchored proteins are found on the plasma membrane of the cell, except for GP-2 which is anchored to the pancreatic zymogen granule, and two proteins found in adrenal chromaffin granules [63,64]. It is likely that more PI-anchored proteins will be found on other cell membranes, but probably not on the cytoplasmic side of the membrane [48].

The presence of a PI anchor on a protein is usually detected by release of the protein by PI-PLC, and after cleavage an epitope on the anchor called cross-reacting

determinant (CRD) can be tested for [48,65]. The anchor can also be identified by biosynthetic labelling experiments, to see what components are incorporated into the anchor [48]. The structure of the anchor renders it susceptible to cleavage by nitrous acid [47]. It is important to look at more than release by PI-PLC for evidence, because some PI anchored proteins are resistant to release [48]. This can be due to a variety of factors [52]. AChE on human erythrocytes is resistant due to acylation of one of the hydroxyl groups on the inositol ring by palmitic acid which is anchored in the plasma membrane [66].

Other than anchoring the proteins to the membrane, the function of this unusual anchor is unclear, although it seems likely that there is some reason the proteins are anchored this way rather than through a transmembrane segment of the peptide. On polarized epithelial cell lines, the PI anchored proteins are found mostly in the apical domain, so the anchor may be an apical transport signal [67]. The PI anchor gives increased lateral mobility to some proteins [68], while others,such as VSG and PH-20, have a decreased mobility [46]. The cytoplasmic domains of receptors promote clustering into clathrin-coated pits, where receptor-mediated endocytosis occurs, but PI-anchored proteins are excluded from these pits [69]. This may allow the proteins to use a different route for internalization [69]. The susceptibility of the anchor to phospholipases may also be part of the function of the anchor [see section

D]. The proteins can be released from the membrane very rapidly, as in the case of VSG of Trypanosomes, where the surface coat is shed to evade the host immune system [70]. The cleavage by phospholipase would also produce diacylglycerol or phosphatidic acid, which may be second messengers involved in cell regulation [50]. Insulin causes the release of some PI-anchored proteins from cells in culture [71]. Structural modifications of the anchor may determine sensitivity or susceptibilty to release [52].

D. Phospholipases

Proteins anchored by PI can usually be released by certain phospholipases. The phospholipases studied so far that do this include bacterial and mammalian PI-PLC, a glycan-specific PI-PLC from Trypanosoma brucei, and PI-phospholipase D (PI-PLD) from a variety of sources. The sites of cleavage for PLC and PLD are shown in figure 1.4 [50,72].

The higher plant and animal forms of PI-PLC require Ca^{2+}, and can vary in the specificity for the type of PI they will cleave (eg PI,PIP,PIP2) [73,74]. The Bacillus PI-PLC enzymes are quite consistent in their release of PI anchored proteins, and therefore are useful to test for the anchor [75]. The PI-PLC from S. aureus shows variation in the sensitivity of releasing PI anchored proteins, so it may be a good tool for finding differences in PI anchored proteins rather than their actual presence [76]. Low and Finean

tested liver plasma membranes with S. aureus PI-PLC and showed that ALP and 5'-nucleotidase (5'-NT) were released [77]. They found no release of phospholipids, and solubilized enzymes were not excluded from Sepharose-6B, so the enzymes did not appear to be released as fragments of the plasma membrane, and they concluded that the enzymes' release was caused by disruption of interactions with PI [77]. Proteins cleaved by PI-PLC lose their hydrophobicity and are released from the membrane [78].

When using the release of proteins by PI-PLC as a tool for detecting PI anchorage, it is important to keep the following in mind. The PI-PLC should be pure, so release is due to PI-specific PLC, and not proteases or other phospholipases. The released protein may actually be associated with another protein that is PI anchored. Even if a protein is not released by PI-PLC, it may still be anchored by PI but resistant to cleavage [76].

A PLC specific for glycan-PI has also been detected in liver plasma membranes [79]. It is Ca^{2+} independent and activated by thiol [79]. This enzyme may be important for the actions of insulin [80].

Malik and Low found a phospholipase in placental tissue that was activated by butanol extraction at low pH. The phospholipase was Ca^{2+} dependent, produced phosphatidic acid, and was identified as a PI-PLD [81].

A glycan-specific PI-PLD has been identified in human serum [80,82,83]. It is Ca^{2+} dependent, and heat labile

[80,82,83]. It does not work on membrane-bound enzymes, but does cleave phosphatidic acid from these enzymes when they are solubilized with detergent [80,82,83]. A glycan-specific PI-PLD has also been found in plasma membranes from human liver [84]. It was unable to release membrane-bound ALP, but converted solubilized enzyme to a hydrophilic form. It was heat stable, had an acid pH optimum, was stimulated by butanol, and had a M_r of 120 000 [84]. It appears to be the same enzyme as the one in serum [84].

E. Liposomes

Synthetic phospholipid bilayers or liposomes were first described by Bangham in the 1960s, they have been used for many applications [85,86,87]. They are classified as multilamellar vesicles (MLV), small unilamellar vesicles (SUV), or large unilamellar vesicles (LUV) [88,89,90]. The distinction between SUV and LUV is not standardized, but SUVs are sometimes considered to be <1000 Å in diameter, with LUVs being larger than this [91]. Liposomes became a useful system for studying membrane processes such as reconstitution of membrane transport proteins and enzyme activity, and for membrane fusion [90]. Because liposomes are versatile, non-toxic and biodegradable, they have been studied for use in drug delivery [87,92,93,94]. There are many ways to form liposomes, including thin film hydration, ultrasonic irradiation, solvent injection, ether injection, detergent removal, reverse phase evaporation, and the

extrusion method [for reviews, see ref. 88,91,95]. In thin film hydration, lipids are deposited from organic solvent to a thin film, and then suspended in aqueous buffer, where they spontaneously form MLV [86]. To make smaller, more uniform MLV, brief sonication, vigorous vortexing, or extrusion through polycarbonate membranes can be used [91]. SUV can be formed by sonicating the MLV to a clear suspension [96] or by removal of detergents from phospholipid-detergent mixtures [97]. Another method which makes unilamellar vesicles is by dissolving lipid in ethanol and injecting into aqueous solution [98]. MLV put through a French press at high pressures more than once become SUV [99]. Lipids dissolved in ether, injected into aqueous solution at 55-65°C or under reduced pressure at 30°C, become LUV when the solvent is evaporated [100]. LUV are also formed by reverse phase evaporation. Phospholipids are mixed with organic solvent, and then the aqueous phase is added. The mixture is sonicated, and then the organic phase is removed under pressure [101]. Vesicles can be characterized for size, number of lamellae, and trapped volume by a number of methods [88,90,91]. The variety of liposomes, and their likeness to natural membranes, make them useful for both a drug delivery system and a model membrane system.

F. References

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Chapter 2

Properties Of Amphiphilic And Hydrophilic Forms Of Alkaline Phosphatase From Human Liver

A. Introduction

ALP from liver is found in a hydrophilic dimer structure in serum, and in this same form when released from the outside of liver plasma membrane by phospholipases or proteases [1,2,3]. The enzyme is anchored to the plasma membrane by a glycosyl-PI structure [1,4,5,6,Chapter 1.C]. When ALP is solubilized from the membrane with the nonionic detergent Triton X-100 or with butanol at high pH, the PI anchor remains covalently attached to the C-terminal amino acid residue of the enzyme and it is amphiphilic [1]. This form of the enzyme has an apparent M_r weight in the presence of Triton X-100 of approximately twice that of the hydrophilic dimer without the PI on gradient gel electrophoresis and on gel permeation chromatography [1,3,Chapter 3.C.5]. Such apparent high M_r values have also been reported with other isoenzymes of ALP from human fetal intestinal and placental tissues [7]. This migration is probably due to the hydrophobic anchor, rather than an actual higher M_r [8]. In this chapter several catalytic and physical properties of the amphiphilic and hydrophilic forms of liver ALP are compared.

B. Materials and Methods

1. Materials

Tris, leupeptin, pepstatin A, 2-amino-2-methyl-1-propanol (AMP), p-nitrophenylphosphate (pNPP), phenylmethylsulfonylfluoride (PMSF), albumin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, homoarginine, phenylalanine, and phenylalanylglycylglycine were purchased from Sigma (St. Louis, MO); phenyl-Sepharose from Pharmacia (Uppsala, Sweden); Triton X-100 from Boehringer Mannheim (Germany); ethylaminoethanol (EAE) from Aldrich (Milwaukee, WI); sodium dodecyl sulfate (SDS) from Bio-Rad (Richmond, CA); Immulon removawells from Fisher Scientific (Fair Lawn, NJ); all other chemicals were from BDH (Toronto, Ont.).

2. Purification of Alkaline Phosphatase

The amphiphilic form of the enzyme was purified as described [1], with the following changes: to recover the aqueous layer after butanol extraction, two centrifugation steps were used. The first was for 25 min at 9000 x g at 4°C. The aqueous layer was then centrifuged at 140 000 x g at 4°C for 45 min and collected. The enzyme was applied to a 1.6 x 20 cm column of phenyl-Sepharose equilibrated in 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, 1 μmol/L leupeptin, 20 μmol/L pepstatin A, 0.1%(w/v) PMSF, pH 8.5. The elution buffer was 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 0.1

mmol/L $ZnCl_2$, 5%(v/v) Triton X-100, pH 8.5. Fractions containing ALP activity were pooled and applied directly to a column of an immobilized monoclonal antibody (mAb) directed against the enzyme [1]. The ALP in the pool from this column was concentrated by adsorption to a 1 mL batch of phenyl-Sepharose at 4°C. The enzyme was eluted with 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, 5%(v/v) Triton X-100, pH 7.5.

Purification of the hydrophilic form is described in Chapter 3.B.2.

3. Enzyme Assays

Alkaline phosphatase activity was assayed in 1 mL of 10 mmol/L pNPP, 1.5 mmol/L Mg^{2+}, 1.0 mol/L EAE, pH 10.3 at 30°C. The increase of absorbance at 404 nm was monitored on a Varian 2200 or Perkin Elmer Lambda 3A spectrophotometer. One unit of enzyme activity was expressed as 1 μmol p-nitrophenol released/min/L [9].

4. Electrophoresis

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was carried out as described, using 7%(w/v) acrylamide [10].

5. Kinetics

Substrate K_m values for pNPP were determined using pNPP concentrations of 1.0-14.3 mmol/L in 1.0 mol/L EAE, pH 10.3,

or a range of 0.002-1.0 mmol/L in 0.782 mol/L AMP, pH 9.0. Both buffers also contained 1.0 mmol/L $MgCl_2$. The K_m values and errors were determined using a computerized Wilkinson plot [11].

To each form of ALP at 300 U/L, one of several phospholipids or albumin was added to 1 mg/mL, and incubated for 30 min at 30°C for the phospholipids, and for 1 h at 22°C for albumin. Phospholipids were suspended by sonication for 15 min in a bath sonicator (Beckman, model 577). Buffer was added to the controls and results were expressed as a percentage of control activity.

Using the assay conditions described above, the hydrophilic and amphiphilic forms of liver ALP were assayed in the presence of the following tissue-specific inhibitors (concentration): phenylalanylglycylglycine (1.0 mmol/L), phenylalanine (2.5 mmol/L), homoarginine (10 mmol/L).

6. Stability to Heat and Sodium Dodecyl Sulfate

The stability to heat was assessed using either of the enzymes at 300 U/L in 100 mmol/L Tris, 100 mmol/L NaCl, 0.1 mmol/L $MgCl_2$, 0.02 mmol/L $ZnCl_2$, pH 7.6, incubating at 56°C and monitoring the activity over 1 h. To assess the stability to SDS, 300 U/L of enzyme in 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, pH 7.6 was incubated at 40°C in the presence of 1%(v/v) SDS, and activity was monitored over 3 h.

7. Reaction with Monoclonal Antibody

The relative affinity of the hydrophilic and amphiphilic forms of ALP for 3 mAbs (OST6, OST7, OST15) [17] was evaluated in microtitre plates as described in Chapter 4.B.5. Binding was expressed as a percentage of a positive control obtained by not washing off the unbound antigen.

8. Conversion of Amphiphilic Alkaline Phosphatase to a Hydrophilic Form by Phosphatidylinositol Phospholipase C

Amphiphilic liver or placental alkaline phosphatase was treated with 2 μg/mL PI-PLC at 30°C, and the amount of conversion to hydrophilic dimer measured over 24 h. The liver enzyme was in 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 0.02 mmol/L $ZnCl_2$, pH 7.6, and the amount of hydrophilic enzyme produced was assayed using a small batch octyl-Sepharose bead assay [2]. The placental enzyme was in a buffer of 150 mmol/L NaCl, 0.1 mmol/L $MgCl_2$, 0.01 mmol/L zinc acetate, 10 mmol/L HEPES, pH 7.0. The amount of hydrophilic enzyme produced in this case was measured using Triton X-114 phase partitioning as described by Malik and Low [13]. Aliquots were assayed at various time intervals. Controls had no PI-PLC added.

C. Results

1. Subunit Molecular Weight

Electrophoresis of both purified hydrophilic and amphiphilic ALP on SDS-PAGE showed one band of stained protein with a M_r of 77 403 ± 5129 [1](gels not shown).

2. Kinetics

At pH 9.0 in 0.782 mol/L AMP, the K_m for pNPP was 0.020 mmol/L ± 0.004 for both the hydrophilic and amphiphilic forms, and at pH 10.3 in 1 mol/L EAE was 4.48 mmol/L ± 0.29 for the hydrophilic and 5.02 mmol/L ± 0.34 for the amphiphilic form.

All four of the phospholipids tested produced a one- to three-fold stimulation of the hydrophilic ALP, with the anionic phospholipids phosphatidylserine and phosphatidylinositol being more effective than the zwitterionic phospholipids phosphatidylcholine and phosphatidylethanolamine (Fig. 2.1). In contrast, the amphiphilic ALP was stimulated only about one-fold by phosphatidylserine, the other three phospholipids being ineffective. Albumin also stimulated the hydrophilic ALP about three-fold but the amphiphilic enzyme only slightly.

The tissue- and isoenzyme-specific inhibitors phenylalanylglycylglycine, phenylalanine and homoarginine had indistinguishable effects on the hydrophilic and amphiphilic forms of liver ALP (results not shown).

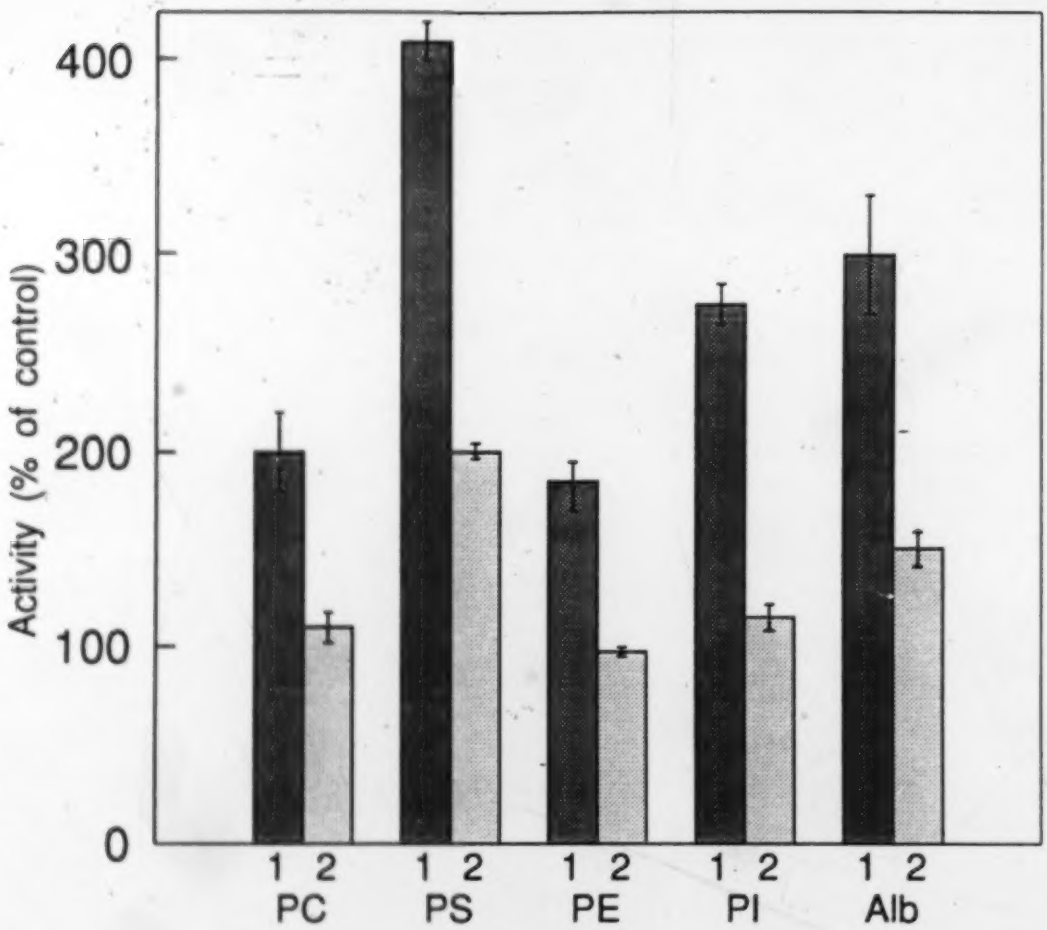

Fig. 2.1 Effect of various phospholipids and albumin on forms of liver alkaline phosphatase: 1, hydrophilic; 2, amphiphilic; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Alb, albumin. Bars represent a range of at least three determinations.

Table 2.1 Pseudo-first-order rate constants (k) for inactivation of alkaline phosphatase by heat and sodium dodecyl sulfate.

Enzyme form	k (s^{-1})(x 10^4)	
	Heat	SDS
Hydrophilic	$\bar{x}$=6.0 SD=0.69 n=5	$\bar{x}$=1.1 SD=0.17 n=3
Amphiphilic	$\bar{x}$=11.4 SD=1.4 n=5	$\bar{x}$=1.4 SD=0.21 n=3

3. Effects of Heat and Sodium Dodecyl Sulfate on Activity

The amphiphilic form of liver ALP showed greater heat lability than did the hydrophilic form (Table 2.1), with the pseudo-first order rate constant being intermediate between the hydrophilic liver isoform of ALP and the more labile hydrophilic bone isoform (not shown). There was no difference in the sensitivity to SDS of the amphiphilic and hydrophilic forms of liver ALP (Table 2.1).

4. Reaction with Monoclonal Antibody

Amphiphilic ALP consistently had a higher affinity than the hydrophilic form for three different mAbs (Table 2.2). Close to twice as much amphiphilic form bound to OST 6, OST 7 and OST 15.

5. Conversion of Amphiphilic Alkaline Phosphatase to a Hydrophilic Form by Phosphatidylinositol Phospholipase C

Amphiphilic liver and placental enzymes were converted to hydrophilic forms at similar rates (Fig. 2.2). No conversion occurred in samples without PI-PLC.

D. Discussion

Amphiphilic ALP solubilized from liver plasma membranes with detergent or butanol at high pH has its PI membrane anchor covalently attached to the C-terminal amino acid residue and has an electrophoretic mobility in gradient polyacrylamide gel electrophoresis (plate 4.1) and an

Table 2.2 Relative affinity for mAbs of two forms of liver alkaline phosphatase

Enzyme form	OST6	OST7	OST15
Hydrophilic	$\bar{x}$=39.2% SD=7.8 n=11	$\bar{x}$=35.6% SD=8.9 n=4	$\bar{x}$=30.6% SD=16.0 n=4
Amphiphilic	$\bar{x}$=66.6% SD=12.1 n=11	$\bar{x}$=61.1% SD=19.9 n=4	$\bar{x}$=64.1% SD=28.1 n=4

Binding expressed as a percentage of a positive control obtained by not washing off the unbound antigen.

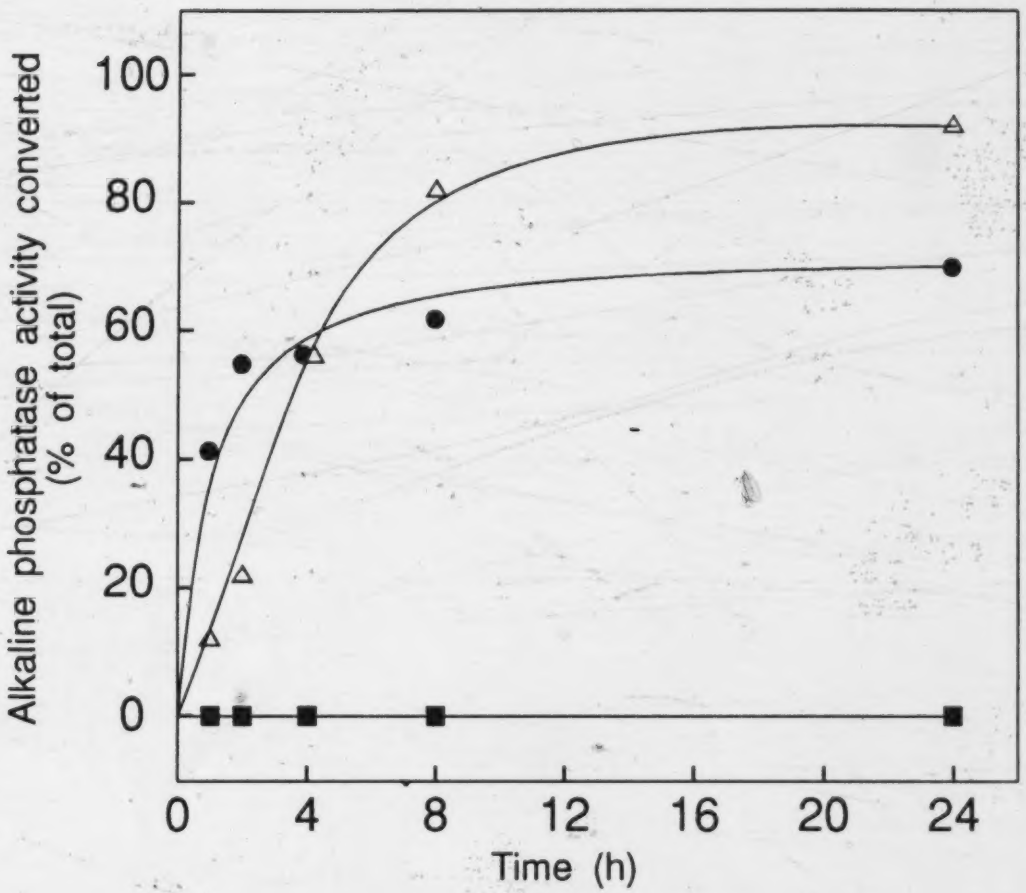

Fig. 2.2 Conversion of amphiphilic alkaline phosphatase to a hydrophilic form by PI-PLC. Purified amphiphilic enzyme from liver (●) or placenta (△) was treated with 2.0 µg/mL PI-PLC. Controls had no PI-PLC (■).

elution volume on gel permeation chromatography equivalent to an apparent M_r of twice that of the hydrophilic dimer [1]. This could be caused by aggregation of two dimers, binding of detergent, or an anomalous migration caused by the anchor. Further evidence indicates that the PI anchor is causing anomalous migration, and that the enzyme is really an unaggregated dimer [8].

There was no difference between the two forms in their substrate K_m values, stability to SDS, or the effect of enzyme inhibitors. However, the amphiphilic form is more sensitive to heat inactivation of catalytic activity, and the hydrophilic form is significantly more activated by hydrophobic compounds such as phospholipids and albumin. These materials may simply be accomplishing what the attached PI already brings about in the amphiphilic form. We have noted that it is very difficult to completely rid the amphiphilic form of albumin during purification.

Another difference noted is that the amphiphilic form had a greater affinity for three different mAbs than did the hydrophilic form; the hydrophobic nature of the membrane anchor present in the amphiphilic form may account for this by causing non-specific binding.

The amphiphilic enzyme can be converted to the hydrophilic form by PI-PLC. The rate of this conversion is compared to other forms of the enzyme in Chapter 3.

The differences in physical, chemical, kinetic and immunological properties between the hydrophilic and

amphiphilic forms of human liver ALP are most likely a result of the covalent attachment of the hydrophobic PI.

E. References

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Chapter 3

Incorporation Of Human Liver And Placental Alkaline Phosphatases Into Liposomes And Membranes

A. Introduction

ALP is one of many proteins that bind to the membrane by attaching covalently to glycosyl-PI [1-7,Chapter 1.C]. Human liver ALP exists in hydrophilic or amphiphilic forms, depending on the method of solubilization from the plasma membrane [6,Chapter 2.D]. The PI anchor in the amphiphilic form may allow the ALP to be incorporated into synthetic liposomes or into red cell ghosts. This membranous form of the enzyme could then be compared to the soluble amphiphilic enzyme with respect to substrate K_m, stability in heat and SDS, effect of inhibitors to ALP, and activation by various phospholipids and albumin.

B. Materials and Methods

1. Materials

Phospholipase C (Bacillus cereus) type III, Triton X-100, pNPP, naphthol AS-MX phosphate, AMP, dipalmitoyl phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phenylalanylglycylglycine, phenylalanine, homoarginine, bromelain, leupeptin, pepstatin A, PMSF, albumin, ovalbumin, orosomucoid, high density lipoprotein (HDL), and low density lipoprotein (LDL) were purchased from Sigma (St. Louis,MO); EAE from Aldrich (Milwaukee, WI); octyl-Sepharose CL-4B from

Pharmacia (Uppsala, Sweden); SDS and gradient gel electrophoresis reagents from Bio-Rad (Richmond, CA). All other chemicals were reagent grade from Fisher Scientific (Fairlawn, NJ).

2. Purification of Enzymes

In the purification of hydrophilic liver ALP, the low pH of 6.5 during tissue extraction allows endogenous phospholipases to release the membrane-bound enzyme as a hydrophilic dimer [8]. The initial steps including butanol treatment of the homogenate and acetone fractionation were carried out as described [9]. After acetone fractionation the enzyme was adsorbed to a column of immobilized monoclonal antibody [6], equilibrated in 10 mmol/L Tris, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, pH 7.6. After washing, the ALP was eluted with 0.1 mol/L AMP at pH 10.3. The flow rate was 0.5 mL/min. The enzyme fraction was dialyzed against the above mentioned Tris buffer and concentrated in an Amicon stirred cell with a PM-10 membrane. Amphiphilic ALP was removed by adsorption to octyl-Sepharose equilibrated in the Tris buffer; the enzyme (about 15 mL) was mixed for 10 min at 22°C with 0.5 mL of packed octyl-Sepharose. This was then centrifuged for 10 min at 10 000 x g, and the supernatant containing hydrophilic liver enzyme was recovered.

Amphiphilic enzyme from human liver was prepared as previously described [6].

Hydrophilic ALP from placenta was purified from a crude commercial preparation (Calbiochem). The sample was dialyzed for 18 h against 'phosphonate' buffer (10 mmol/L MES, 100 mmol/L NaCl, 1.0 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, 0.01% (w/v) PMSF, 1 μmol/L leupeptin, and 1 μmol/L pepstatin A, pH 6.0). Following adsorption to an immobilized phosphonate and elution with 50 mmol/L pNPP in phosphonate buffer, the pH was raised to 7.6 by the addition of 100 mmol/L Tris, pH 9.0, and concentration carried out in an Amicon cell with a PM-10 membrane [10]. The final step was gel filtration on a Sepharose CL-6B, K25/100 column. The buffer was 10 mmol/L Tris, 300 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, pH 7.6 [10].

Amphiphilic placental enzyme was purified from frozen human placenta as described [11].

Phosphatidylinositol-specific phospholipase C (PI-PLC), was purified from a commercial preparation of non-specific phospholipase C [12].

3. Isolation of Plasma Membranes and Assay for Alkaline Phosphatase

Plasma membranes were prepared from human liver [13]. ALP activity was assayed as described in Chapter 2.B.3.

4. Preparation of Liposomes and Red Cell Ghosts, and their Incorporation with Alkaline Phosphatase

Dipalmitoylphosphatidylcholine (30 mg) was dissolved in 5 mL of chloroform, and dried in a rotary evaporator for 18 h. A buffer made of 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 20 μmol/L $ZnCl_2$, pH 7.6 (5 mL) was then added and the solution was sonicated for 15 min in a bath sonicator (Beckman, model 577). The liposomes were stored at 4°C until used. Red cells were obtained from fresh whole blood and ghosts made as described [14]. Red cell ghosts have no endogenous ALP activity.

Equal volumes of liposomes (6 mg lipid/mL) or red cell ghosts (1 mg protein/mL) and ALP (1000 U/L), in the Tris buffer described above, were mixed and incubated most often at 22°C. In later experiments incorporation was found to be faster at higher temperatures, and 44°C was used. At timed intervals, 100 μl aliquots were removed and centrifuged in a Beckman airfuge at 140,000 x g for 10 min [6]. The total activity before centrifugation and the activity in the supernatant were assayed and used to calculate the percent incorporation. It could be shown that activity not in the supernatant was in the liposomes or ghosts.

5. Release by PI-PLC of Alkaline Phosphatase from Plasma Membranes or Liposomes

Liver membranes with ALP at 300 U/L, or liposomes or red cell ghosts into which amphiphilic liver or placental enzyme

had been incorporated to 200 U/L, were incubated at 30°C with various concentrations of PI-PLC. The buffer used was 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 20 µmol/L $ZnCl_2$, pH 7.6, and the protease inhibitors leupeptin (1 µg/mL), pepstatin A (1 µg/mL), and PMSF (0.02% (w/v)) were added. At timed intervals 100 µL of sample was removed and centrifuged as described for incorporation. Total activity and supernatant were assayed, and the percent activity released was calculated.

To allow a semiquantitative comparison of the relative resistance to PI-PLC for the release of various membrane forms of ALP, and for the conversion of amphiphilic to hydrophilic enzyme, the time to release (convert) 50% of the enzyme by 1 µg/mL of PI-PLC was determined from the time-course experiments.

The mAb used was purified as described [15]. The protein concentration of the solution of mAb was 3.7 mg/mL, and it was stored at -70°C. The binding to monoclonal antibodies was also tested on microtitre plates, using the method described in Chapter 4.B.5.

6. Triton X-100, Butanol, or Protease Treatment of Plasma Membranes

Triton X-100 was added to plasma membranes to 1%(v/v), or an equal volume of 1-butanol was mixed with membranes, at 4°C for 30 min. Treatment with the protease, bromelain, at 5 mg/mL was for 18 h at 30°C. The membranes were

centrifuged at 12 000 x g for 10 min, and the solubilized enzyme assayed.

7. Gradient Gel Electrophoresis and Octyl-Sepharose Adsorption

A gradient of 4% to 26% polyacrylamide in a slab was used. The 4% solution contained 3.84 mL of acrylamide, 0.16 mL of bis-acrylamide and 1.0 mL of sucrose. This was made up to 100 mL with electrode gel buffer (88 mmol/L Tris, 2.5 mmol/L disodium EDTA, 81 mmol/L boric acid, pH 8.28). The 26% solution contained the same buffer, plus 24.96 mL of acrylamide, 1.04 mL of bis-acrylamide, and 4.0 mL of sucrose. For each gel approximately 16 mL of 26% and 16 mL of 4% acrylamide was used. These solutions were put into a gradient mixer, and to each was added 5 µL of TEMED, 100 µL of 10% (w/v) ammonium persulfate, and Triton X-100 to 0.1% (v/v). The gradient was made using a Gilson minipulse 2 pump at setting #8 with medium tubing into a glassplate mold. The gel was then overlayed with 50% ethanol and left to polymerize for 15 min. The ethanol was washed off and wells were made using 10 mL of 4% polyacrylamide gel solution, 100 µL of 10% ammonium persulfate and 5 µL of TEMED. A sample volume of 100 µL containing a grain of sucrose and 0.1% (v/v) Triton X-100 was applied to the wells and the gel electrophoresed for 18 h at a constant current of 40 mA. Bands of ALP activity were visualized using a substrate mixture (3 mg/mL naphthol AS-MX phosphate,

1.5 mmol/L Mg^{2+}, in 1 mol/L EAE, pH 10.3) that resulted in a fluorescent product.

Adsorption to octyl-Sepharose was carried out in 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 20 mol/L $ZnCl_2$, pH 7.6 as described [8].

8. Kinetics and Stabilities

The substrate K_m values in 1.0 mol/L EAE, 1.0 mmol/L $MgCl_2$, pH 10.3, the activation by phospholipids and albumin, the effect of tissue-specific inhibitors, and the stability to heat and SDS were carried out as described in Chapter 2.B.5 and 2.B.6.

C. Results

1. Incorporation of Alkaline Phosphatase into Liposomes and the Effect of Hydrophobic Proteins

Amphiphilic liver and placental ALP incorporated into liposomes in a time dependent manner, with a fast initial rate, followed by a slower rate (Fig. 3.1). Purified hydrophilic liver or placental enzymes did not incorporate, nor did liver enzyme released from plasma membranes by PI-PLC or bromelain. Liposomes containing incorporated ALP, when solubilized with 1% (v/v) Triton X-100 and assayed for enzyme activity, had the same catalytic activity as did the intact liposomes. Therefore, it is unlikely that enzyme was

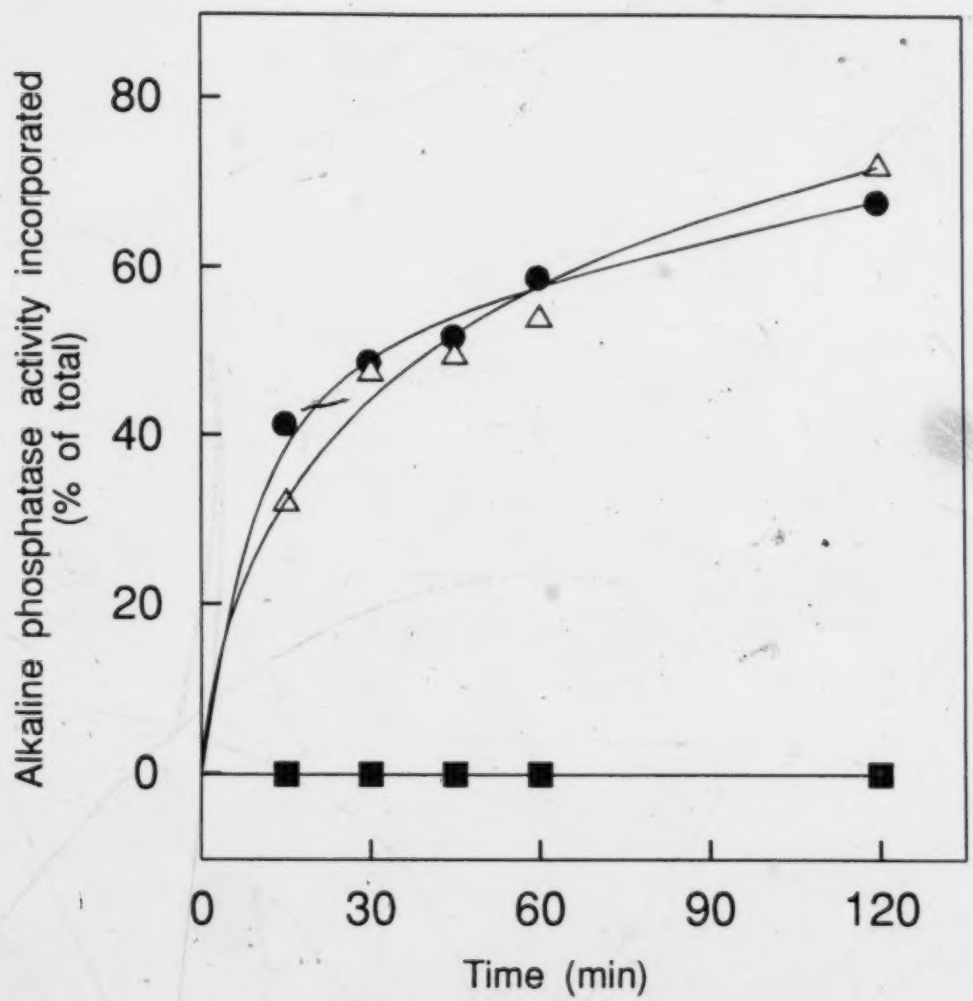

Fig. 3.1 Incorporation of liver or placental alkaline phosphatase into liposomes. Liposomes (6 mg/mL) and enzyme (1000 U/L) were incubated at room temperature and samples were assayed before and after centrifugation. Activity not in the supernatent was shown to be incorporated: ●, amphiphilic liver; △, amphiphilic placenta; ■, purified hydrophilic liver or placenta, or enzyme released from plasma membranes by PI-PLC or bromelain.

trapped within the lipid bilayer, inaccessible to substrate. Any change in enzyme activity on incorporation into liposomes was minor (<10%).

In the presence of albumin, orosomucoid, HDL, or LDL, incorporation was inhibited, while ovalbumin had no effect (Fig. 3.2). The most potent protein inhibitors of incorporation were LDL and HDL, where at 1 mg/mL these proteins inhibited incorporation by more than 85%. Enzyme already incorporated into liposomes could not be released by 5 mg/mL albumin or by 1 mol/L NaCl. These data are consistent with the incorporation of ALP into liposomes via PI.

2. Release by PI-PLC of Alkaline Phosphatase from Plasma Membranes or Liposomes

ALP was released by PI-PLC from both liver plasma membranes and liposomes incorporated with amphiphilic liver or placental enzyme (Figs. 3.3 and 3.4). Release of both enzymes was variable in rate from one batch of liposomes to another. The rates of release from liposomes of liver or placental enzyme were similar but about 65X slower than for the liver enzyme in plasma membranes. The rate of release from liposomes was PI-PLC concentration dependent and 100% release could be achieved if enough phospholipase was added (Fig. 3.3). No enzyme was released in the absence of PI-PLC. There was no appreciable change in enzyme activity on

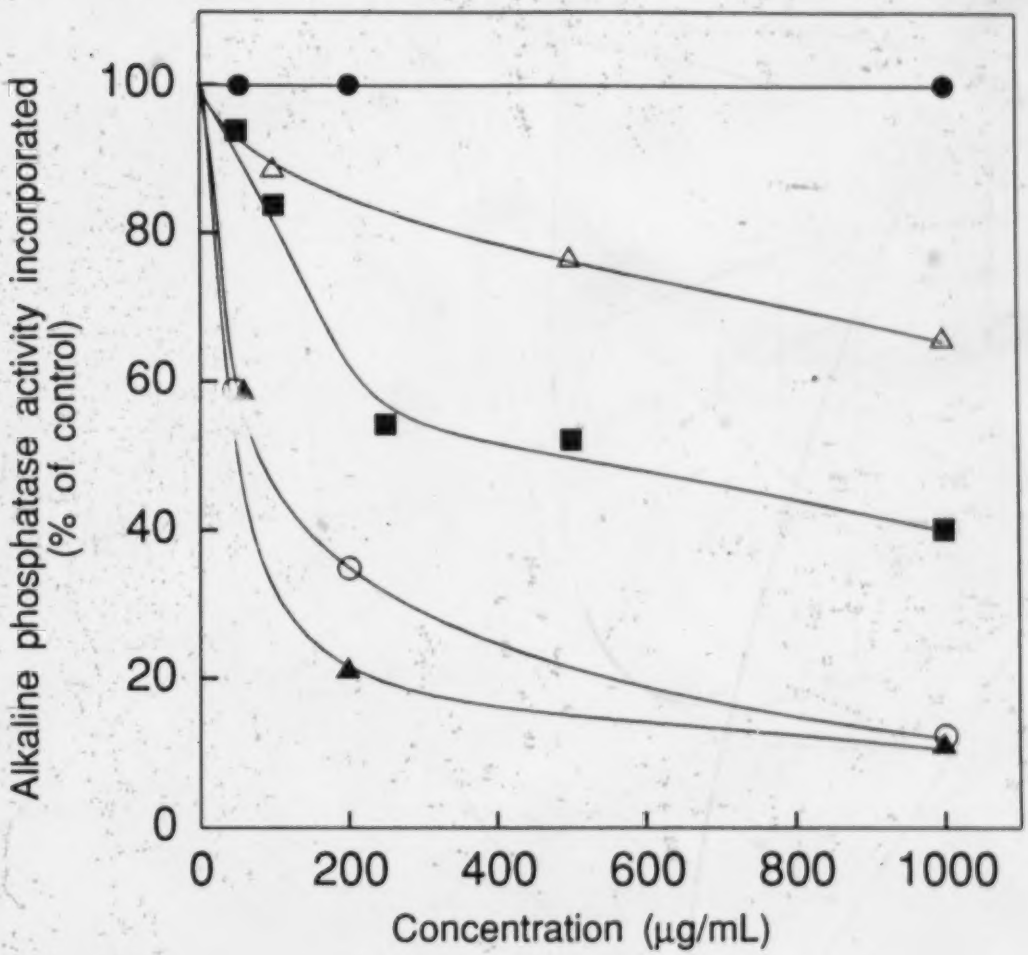

Fig. 3.2 Effect of hydrophobic proteins on incorporation of liver alkaline phosphatase into liposomes. Liposomes (6 mg/mL), amphiphilic liver ALP (1000 U/L) and individual proteins at various concentrations were mixed and incubated at 44 °C for 2 h. Assays were as for figure 3.1. A control with no added proteins was taken as 100% incorporation. The proteins used were: ●, ovalbumin; △, orosomucoid; ■, albumin; ○, LDL; ▲,HDL.

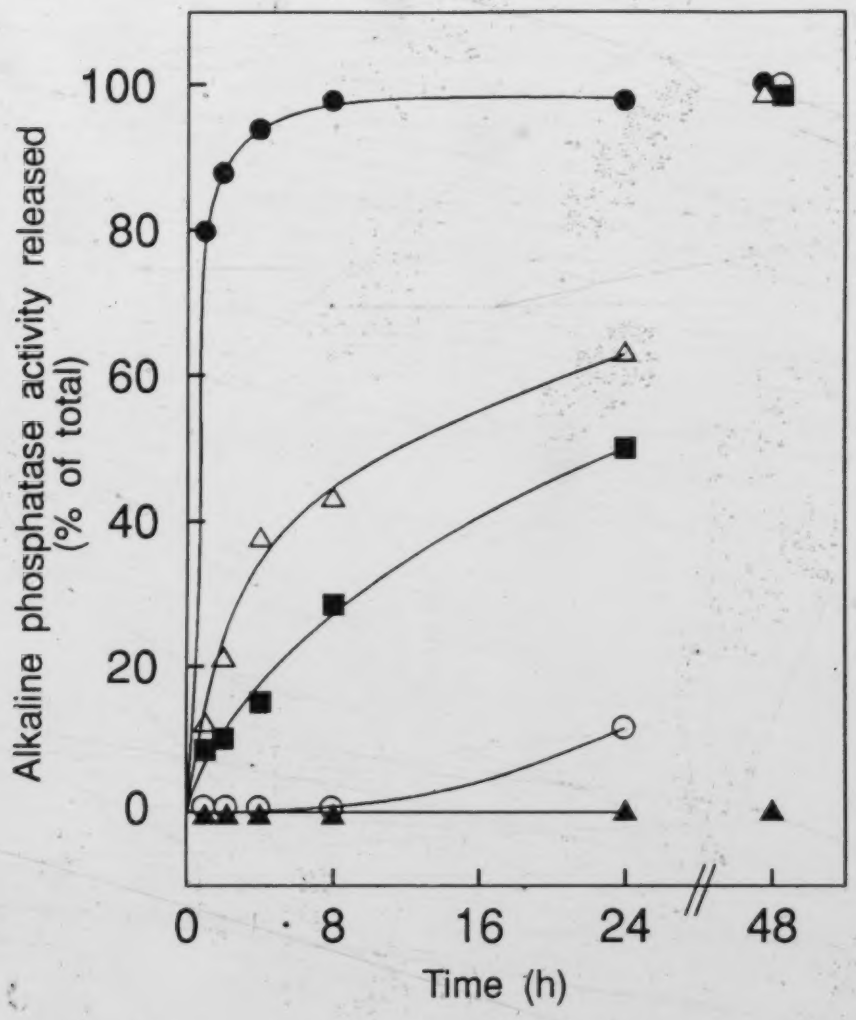

Fig. 3.3 Release by PI-PLC of liver alkaline phosphatase from liver plasma membranes or liposomes. Plasma membranes with 300 U/L ALP were incubated with 20 µg/mL PI-PLC at 30 °C and solubilized activity assayed, ● . Liposomes containing ALP at 200 U/L were incubated at 30 °C with PI-PLC at the following concentrations: △, 20 µg/mL; ■, 2 µg/mL; ○, 0.5 µg/mL; ▲, no PI-PLC. After 24 h PI-PLC at 20 µg/mL was added to all samples and release measured at 48 h.

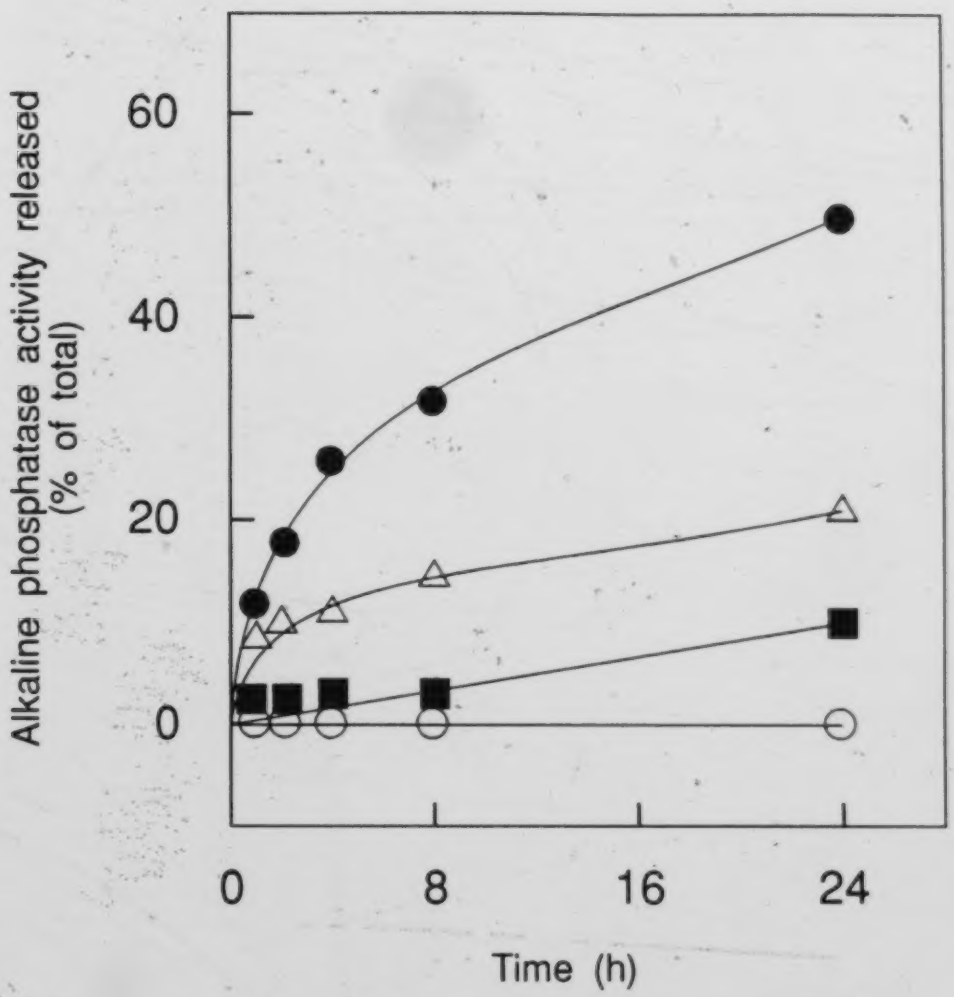

Fig. 3.4 Release by PI-PLC of placental alkaline phosphatase from liposomes. Liposomes containing ALP at 200 U/L were incubated at 30 °C with PI-PLC at the following concentrations: ●, 5 μg/mL; △, 2 μg/mL; ■, 0.5 μg/mL; ○, no PI-PLC.

release by PI-PLC and the ALP was stable over the 48 h incubation.

Release by PI-PLC of ALP from plasma membranes of human liver and red blood cells, from liposomes, or from two cultured osteosarcoma cell lines were semiquantitatively compared (Table 3.1). In general, the ALP in natural membranes was less resistant to PI-PLC than enzyme in synthetic membranes (liposomes); the conversion of amphiphilic liver or placental enzyme to hydrophilic dimers was intermediate in resistance to PI-PLC [12].

3. Incorporation and Release of Alkaline Phosphatases from Red Cell Ghosts

Both amphiphilic liver and placental enzyme incorporated into red cell ghosts (Fig. 3.5), although about 16X slower than their incorporation into liposomes (Fig. 3.1). The hydrophilic forms of the enzyme did not incorporate. The enzyme could also be released from ghosts by PI-PLC (Fig 3.6), but at a faster rate than that from liposomes (Table 3.1). The rates of release of liver and placental enzyme were similar and 100% release could be achieved if enough PI-PLC was added.

4. Effect of Monoclonal Antibody on Incorporation and Release

A mAb to liver ALP inhibited by 50% the extent of incorporation of amphiphilic liver ALP, but not amphiphilic

Table 3.1. Rates of release by PI-PLC of various forms of alkaline phosphatase

Enzyme form	Time to 50% release (conversion) by 1 µg/mL PI-PLC (h)* (Range)	Number of samples
Saos-2 cell suspension**	0.81 (0.42-1.2)	6
KTOO5 cell suspension**	0.85 (0.83-0.87)	6
Liver plasma membranes	2.4 (1-8)	18
KTOO5 cell monolayer**	3.8 (3.3-4.2)	6
Purified placental, amphiphilic	6.0 (2-10)	9
Purified liver, amphiphilic	7.3 (4-10)	12
Saos-2 cell monolayer**	11 (3.8-18)	6
RBC ghosts incorporated with placental ALP	43 (40-44)	6
RBC ghosts incorporated with liver ALP	47 (44-48)	9
Liposomes incorporated with placental ALP	159 (40-350)	15
Liposomes incorporated with liver ALP	162 (114-210)	6

* Release (conversion) was assessed at PI-PLC concentrations from 0.1 to 20 µg/mL. However, the rate of release is linearly related to PI-PLC concentration and all values were standardized to 1.0 µg/mL to allow comparison.

** Calculated from data of Nakamura, Nakamura, and Stinson [16].

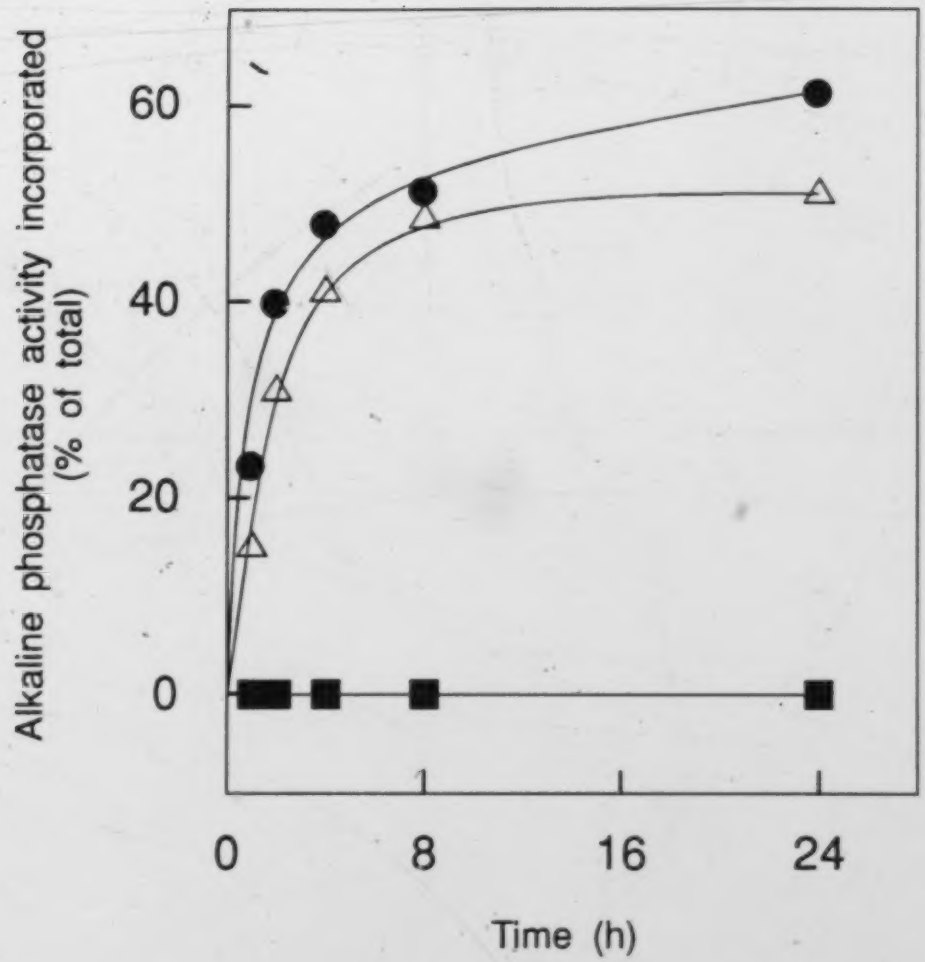

Fig. 3.5 Incorporation of alkaline phosphatase into red cell ghosts. Membranes and liver or placental enzyme (1000 U/L) were incubated at 22 °C, and assayed before and after centrifugation to calculate % incorporation: ●, amphiphilic liver; △, amphiphilic placenta; ■, hydrophilic liver or placenta.

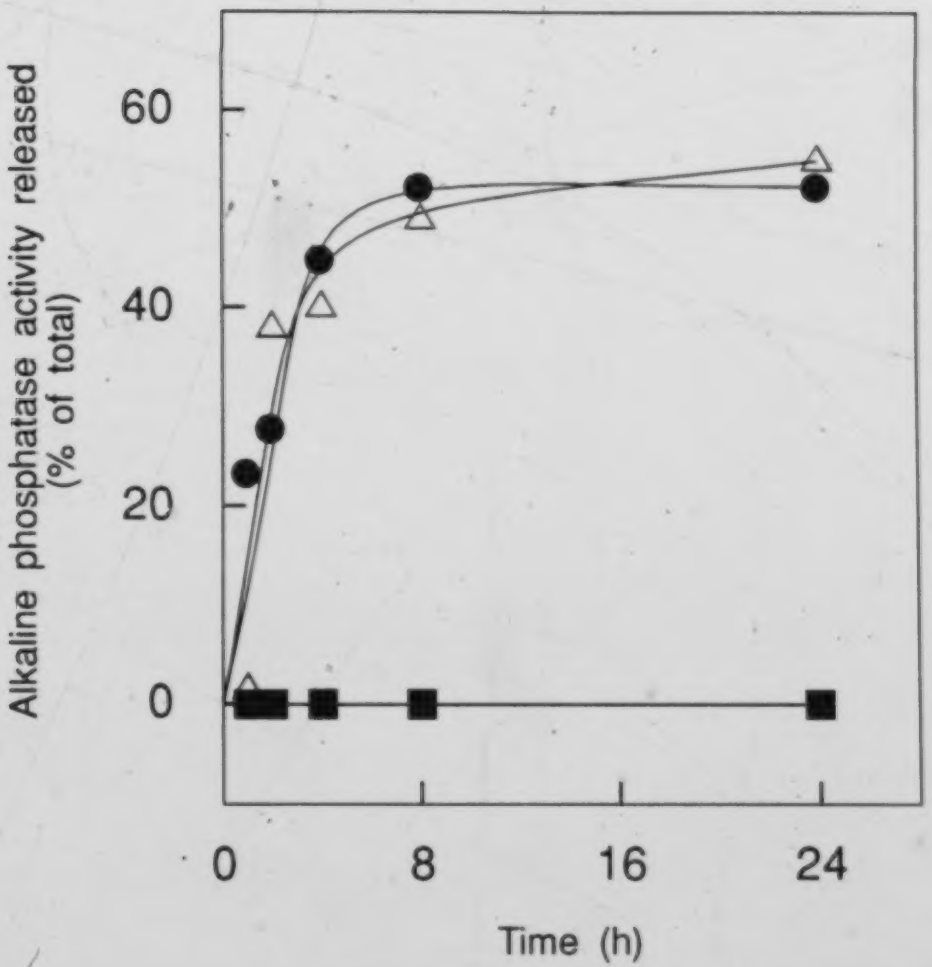

Fig. 3.6 Release of alkaline phosphatase from red cell ghosts. Red cell ghosts containing 200 U/L alkaline phosphatase were incubated with 2 μg/mL PI-PLC at 30 °C: ●, amphiphilic liver; △, amphiphilic placenta; ■, no PI-PLC.

placental enzyme, into liposomes (not shown). The mAb abolished release of liver enzyme from liposomes and the conversion of amphiphilic liver enzyme to a hydrophilic form (Fig. 3.7 and 3.8). There was no effect on the placental enzyme. The mAb decreased the amount of enzyme released from liver plasma membranes (Fig. 3.9). The catalytic activity of the different forms of the enzyme was only slightly effected by the mAb, except for the amphiphilic liver enzyme which was inhibited by 50%.

The liposome-incorporated enzyme did not bind, or stay bound, to the mAb coated on wells, whereas 60% of the amphiphilic form attached.

5. Gradient Gel Electrophoresis and Adsorption to Octyl-Sepharose

Gradient gels of polyacrylamide showed purified amphiphilic liver ALP to have an apparent M_r of about 440,000, and the hydrophilic dimer to be 214,000 [Plate 4.2]. Table 3.2 is a summary of the results from gradient gel electrophoresis, octyl-Sepharose adsorption and incorporation into liposomes, of the ALP formed from various treatments. Amphiphilic forms of the enzyme show near 100% adsorption to octyl-Sepharose, and will incorporate into liposomes (and other membranes). On the other hand, hydrophilic dimeric enzyme does not adsorb to the hydrophobic support, and will not incorporate into membranes.

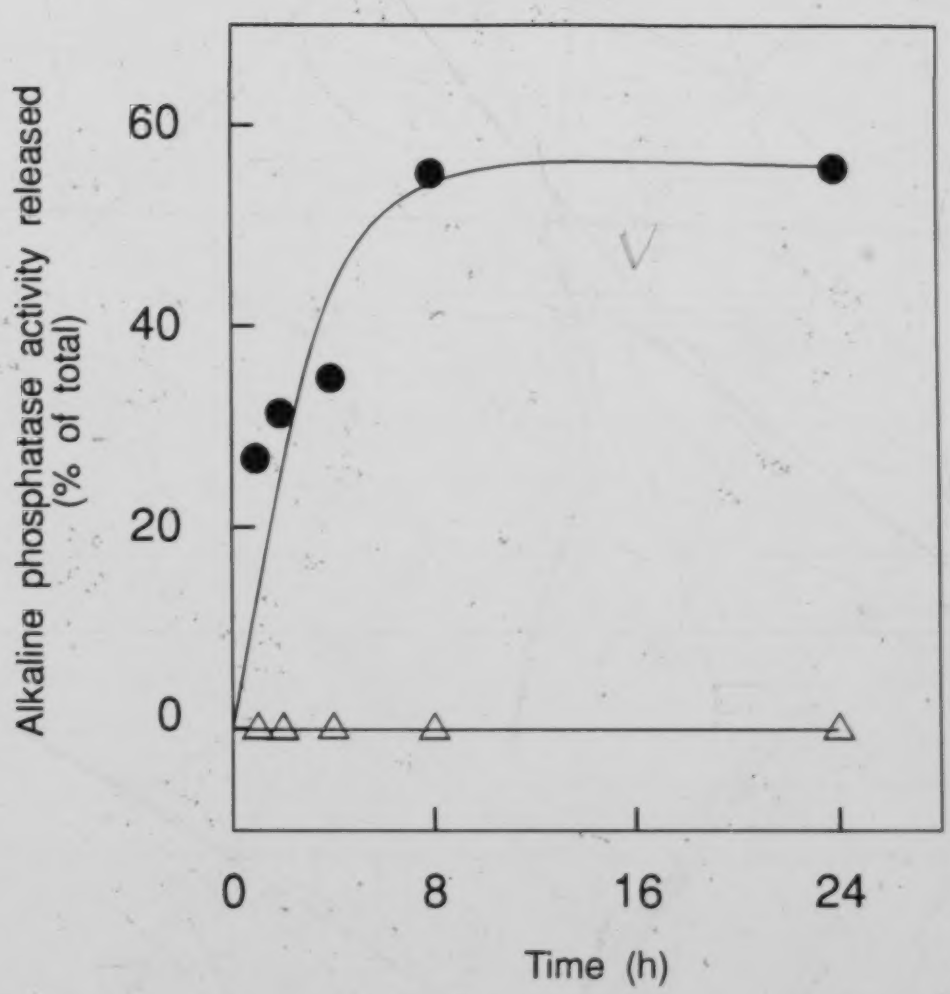

Fig. 3.7 Effect of a mAb to liver alkaline phosphatase on the enzymes' release from liposomes by PI-PLC. The monoclonal antibody, OST 15, was used as a 1/1000 dilution of stock. The amount of activity released was measured by assaying the supernatent after centrifugation. Samples were incubated with 2 µg/mL PI-PLC at 30°C: ●, no antibody present; △, with antibody.

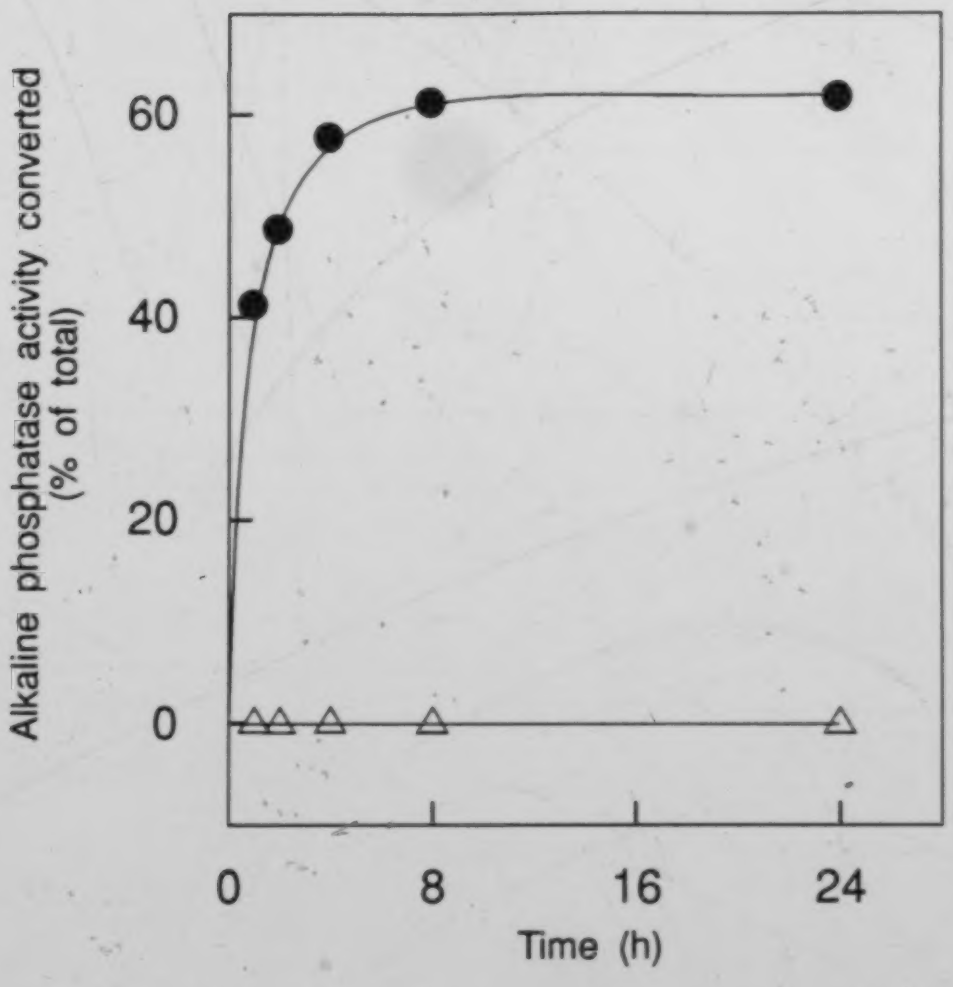

Fig. 3.8 Effect of a mAb to liver alkaline phosphatase on conversion of amphiphilic liver enzyme by PI-PLC. Conditions as described for figure 3.7, except amount of hydrophilic enzyme product was measured after adsorption to octyl-Sepharose: ●, no antibody present; △, with antibody.

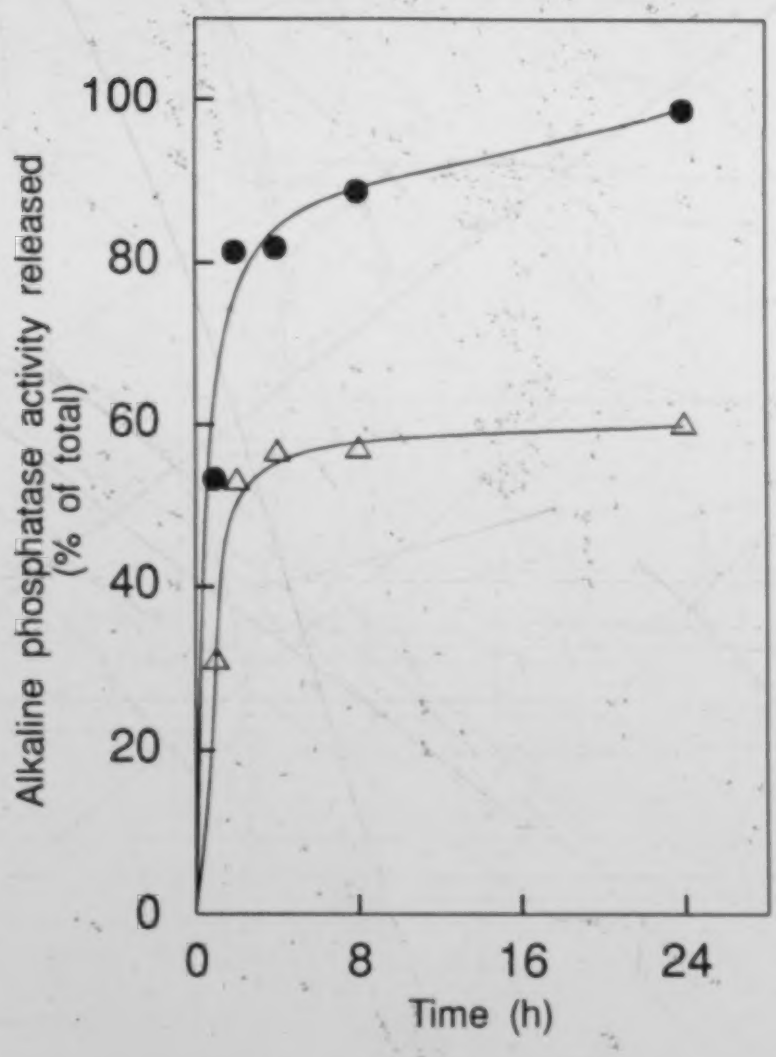

Fig. 3.9 Effect of a mAb to liver alkaline phosphatase on the release of enzyme from liver plasma membranes by PI-PLC. Membranes at 300 U/L were incubated with 2 µg/mL PI-PLC at 30°C: ●, no antibody; △, with OST 15 at a dilution of 1/1000 of stock.

Table 3.2 Properties of solubilized and released forms of alkaline phosphatase from human liver

Enzyme form	Apparent quaternary structure	Incorporation into liposomes	Adsorption to octyl-Sepharose
Triton extract of plasma membranes	Tetramer	+	+
Butanol extract of plasma membranes	Tetramer & Aggregates	+	+
PI-PLC released from plasma membranes	Dimer	-	-
Bromelain released from plasma membranes	Dimer	-	-
Purified hydrophilic	Dimer	-	-
Purified amphiphilic	Tetramer	+	+

6. Kinetics and Stabilities

The K_m values for pNPP were 5.02 mmol/L ± 0.34 for amphiphilic ALP, and 4.57 mmol/L ± 0.55 for amphiphilic enzyme incorporated into liposomes. There was no difference in the activation of the two forms of the enzyme by phospholipids and albumin (for data of amphiphilic ALP see Fig. 2.1). The tissue-specific inhibitors phenylalanylglycylglycine, phenylalanine, and homoarginine had indistinguishable effects on the soluble and liposome-incorporated amphiphilic enzyme. For example, homoarginine inhibited the amphiphilic form to 27.4% ± 2.2 (1 SD), and the liposome incorporated form to 28.7% ± 2.9 (1 SD). The liposome incorporated enzyme also gave pseudo-first-order rate constants for the inactivation by heat and SDS that were in the range of the amphiphilic enzyme (Table 2.2).

D. Discussion

Amphiphilic forms of liver or placental ALP incorporated into liposomes whereas PI-PLC or protease-released hydrophilic dimeric enzyme did not (Fig. 3.1). Thus, incorporation depends on the presence of a hydrophobic nonprotein tail that would be removed by either PI-PLC or a protease. The results obtained from octyl-Sepharose adsorption concur; any process that results in hydrophilic enzyme eliminates the hydrophobicity of the molecules and prevents adsorption (Table 3.2). ALP incorporated into liposomes can be released from these liposomes by PI-PLC, in

the same way it can be released from plasma membranes (Fig. 3.3). The PI-PLC removes a diacylglycerol molecule that is covalently attached to the C-terminal of the protein through a glycan moiety [1-7]. This diacylglycerol is the natural membrane anchor for ALP and when solubilization leaves the anchor intact, the ALP remains amphiphilic. Since incorporated ALP can be released to near 100% if sufficient PI-PLC is added, the phosphatase must not penetrate the outer most bilayer of the multilamellar liposome. It is likely that anchorage in the liposomes is by insertion of the covalently attached PI into the bilayer.

Further evidence for this PI anchorage is the inhibition of incorporation by proteins (Fig. 3.2). The more hydrophobic the protein the greater the inhibitory effect. These proteins may interact with the hydrophobic tail on the enzyme, thereby inhibiting incorporation. Another explanation is steric competition by the hydrophobic proteins, which may saturate the surface of the liposome. Medof et al found similar results on the incorporation of decay-accelerating factor into red cells [17].

A mAb to liver ALP had no effect on the incorporation or release of the placental enzyme. Thus the positive effects on the liver enzyme are probably a result of the association between the mAb and the enzyme (Figs. 3.7, 3.8 & 3.9). Release by PI-PLC from liposomes is abolished by the mAb but only inhibited by 40% after 24 h from plasma membranes. It is not clear why there should be this difference but perhaps

the liposome surface allows a closer association between the mAb and the enzyme and prevents access of the PI-PLC to the cleavage site. Conversion by PI-PLC of amphiphilic liver ALP to a hydrophilic form is also prevented by the mAb, and is consistent with a limited access of the phospholipase. The liposome-incorporated enzyme did not react with the mAbs bound to microtitre plates, but since binding seems to have occurred in solution, this was probably due to steric hindrance occuring at the plate surface.

There is a large range for the rates of release by PI-PLC of a number of forms of ALP (Table 3.1). The more natural membranes are least resistant to PI-PLC and this may be important physiologically, since PI-PLC activation, and thus release of membrane enzymes, could be important in membrane signal transduction [18].

There is no significant difference between liver and placental enzymes in either their rates of incorporation or in their rates of PI-PLC mediated release. Presumably M_r and other differences between the proteins (e.g., amino acid sequence, carbohydrates) are not important in their rates of incorporation nor in the ability of the PI-PLC to release them [19].

When the liposome-incorporated enzyme was compared to the soluble amphiphilic form for a variety of different properties, no differences were found. The anchorage to the artificial membrane did not seem to alter any of the

enzyme's kinetics or stabilities, but did affect the rate of conversion by PI-PLC.

In conclusion, amphiphilic ALP from human liver and placenta readily incorporate into both synthetic and natural membranes. Further, these enzymes are released from all membranes by PI-PLC, albeit with differing sensitivities, depending on the membrane but not the source of the ALP. It is likely that the incorporation of ALP into these membranes is via PI and is identical to its anchorage in natural cells.

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Chapter 4

High Molecular Weight Alkaline Phosphatase In Serum Has Properties Similar To The Enzyme In Plasma Membranes Of Liver

A. Introduction

There are different theories as to the origin of the high M_r or 'particulate' form of ALP found in the sera of patients with cholestatic liver disease (Chapter 1.B). To try and elucidate this problem we compared the ALP found in a high M_r form from serum to that form of the enzyme found in plasma membranes from liver, and to a purified hydrophilic dimer of ALP.

B. Materials and Methods

1. Materials

Tris, pNPP, albumin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phenylalanylglycylglycine, phenylalanine, homoarginine, phospholipase D(Type III from peanut), naphthol AS-MX phosphate, 2-[N-Morpholino]ethanesulfonic acid (MES), and Triton X-100 were purchased from Sigma (St. Louis. MO); Immulon 1 removawells from Fisher Scientific (Fair Lawn, NJ); Sepharose CL-6B and octyl-Sepharose CL-4B from Pharmacia (Uppsala, Sweden); EAE (gold label) from Aldrich (Milwaukee, WI); ALKPHOR gels from Quantimetrix (Hawthorne, CA); 5'-NT assay materials from bioMerieux

(Marcy l'Etoile, France); SDS from Bio-Rad (Richmond, CA). All other chemicals were reagent grade from BDH (Toronto, Ont.).

2. Sources of Enzyme and Assay

Hydrophilic and amphiphilic alkaline phosphatases were purified as described in Chapter 2.B.2. Plasma membranes were purified from human liver using a Percoll density gradient [1]. PI-PLC was purified as published, with minor modifications [2]; chromatography on CM-Sephadex was omitted, and gel filtration was included after phenyl-Sepharose chromatography. For gel filtration, an 80 x 2 cm Sephadex G-200 column was used at a flow rate of 0.6 mL/min. The buffer was 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, pH 7.6. Sera with high 5'-NT activity were collected from the hospital laboratory, and screened for high M_r ALP by standard pore polyacrylamide gel electrophoresis (PAGE), using ALKPHOR gels, according to manufacturer's instructions.

Sera containing high M_r or 'particulate' ALP were partially purified by collecting the void volume from gel permeation chromatography on Sepharose CL-6B. The buffer used was 50 mmol/L Tris, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, 100 mmol/L NaCl, pH 7.6. Fractions were concentrated to approximately 300 U/L in an Amicon stirred cell with a PM-10 membrane. Following this,

the particulate ALP was pelleted by centrifugation in a Beckman airfuge at 140 000 x g for 10 min and resuspended in the above buffer without NaCl. This buffer without the salt was used in all experiments described below except where noted otherwise.

ALP activity was assayed as described in Chapter 2.B.3.

The 5'-NT was assayed by an endpoint methodology using materials supplied by bioMerieux, with inosine 5'-monophosphate as the substrate. The product inosine was coupled using nucleoside phosphorylase and xanthine oxidase to form the final product, quinoneimine, which was measured at 500 nm. For assay, 3 µL of sample was added to 250 µL of substrate solution, incubated for 1 h at 30°C, and then 750 µL of distilled and deionized water added.

3. Treatments with Triton X-100, Butanol or PI-PLC, and Electrophoresis

Three different treatments were used to either solubilize or release ALP from its particulate form in sera. Triton X-100 solubilization was accomplished by adding to high M_r or plasma membrane ALP at 200-300 U/L the detergent to 1%(v/v). This was mixed at 4°C for 60 min, and then centrifuged at 140 000 x g for 10 min. For butanol solubilization, an equal volume of the solvent was mixed with ALP at 200-300 U/L for 10 min at

4°C, and then centrifuged at 12 000 x g for 10 min to recover the aqueous layer. PI-PLC was used at 10 µg/mL and 30°C for 4 h to completely release ALP from particulate forms. Samples were then subjected to electrophoresis on gradient or standard pore PAGE, the first mentioned technique as described in Chapter 3.B.7, and the latter as above.

4. Activations, Kinetics and Stabilities

To ALP at 300 U/L from each of the three sources, activation by phospholipids or albumin, K_m values for pNPP in 1.0 mol/L EAE, 1.5 mmol/L $MgCl_2$, pH 10.3, effect of the tissue specific inhibitors phenylalanylglycylglycine, phenylalanine, or homoarginine, and the stability to heat and SDS were determined as described in Chapter 2.B.5 and 2.B.6.

5. Hydrophobic and Monoclonal Antibody Interactions

The hydrophobicity of the three enzyme forms was assessed by incorporation into liposomes and adsorption to octyl-Sepharose. For incorporations the enzyme at 200 U/L was mixed with an equal volume of liposomes (6 mg phospholipid/mL) made from dipalmitoylphosphatidylcholine and mixed for 4 h at 44°C [see Chapter 3.B]. Centrifugation at 140 000 x g for 10 min followed, and the supernatant was assayed for enzyme that did not incorporate. The pellet was

resuspended to analyze the amount of activity that did incorporate. Adsorption to octyl-Sepharose was assessed in a small batch assay [3].

A mAb to liver-type ALP, designated OST6, was raised as described [4]. The mAb (50 µl of 2 µg/mL in 0.4 mol/L $NaHCO_3$, pH 9.4) was coated onto wells by incubation for 18 h at 4°C. Gelatin at 1%(w/v) was used as a blocker. The washing solution was 9.4 mmol/L NaH_2PO_4, 137 mmol/L NaCl, 2.7 mmol/L KCl, 0.05% Tween 20, pH 7.9. ALP (50 µL at 50 U/L) was added to each well and incubated at 37°C for 1 h. The wells were rinsed twice with the washing solution described above and finally with the carbonate buffer to facilitate assay of ALP. Assay medium was as described and after 10 min the reaction was stopped with 1 mol/L NaOH. The yellow product was read at 405 nm in a Titertec Multiscanner. The negative control had no antigen added, and in the positive control the antigen was not washed off. Purified hydrophilic ALP, high M_r ALP, and liver plasma membranes were evaluated.

6. Release of ALP and 5'-nucleotidase by PI-PLC

PI-PLC (0.5 µg/mL final concentration) was added to liver plasma membranes or high M_r ALP at 200-300 U/L in the Tris buffer at pH 7.6, and incubated at 30°C for 24 h. At various times samples were removed, assayed for ALP and 5'-NT as described above, and centrifuged

for 10 min at 140 000 x g. The activities of ALP and 5'-NT in the supernatant were determined. The release of ALP and 5'-NT by PI-PLC was also assessed in partially purified high M_r ALP from 20 individual sera. Samples with 20 to 200 U/L ALP in Tris buffer at pH 7.6 were incubated with 10 µg/mL PI-PLC for 18 h at 30°C, and the amount of release of ALP and 5'NT assessed as above.

7. Treatment with Phospholipase D Enzymes

The release of ALP from a high M_r complex and from plasma membranes was also assessed with a PLD partially purified from human liver [3], with a glycan-specific PLD from serum [5], and with a commercial PLD. Treatments of the two particulate forms as well as their Triton- and butanol-solubilized counterparts were in 50 mmol/L MES, pH 6.0. The PLD from liver (63 µL), as a Triton-solubilized extract of plasma membranes [3], was mixed with 112 µL of each form of ALP (300 U/L) and incubated at 30°C for 4 h. For the particulate forms of the enzyme, the sample was then centrifuged at 12 000 x g for 10 min. The pH of the sample (150 µL) was increased by addition of 100 µL of 250 mmol/L Tris, pH 9.0. An equal volume of butanol was added and mixed for 10 min to remove the triton, and octyl-Sepharose beads were added to the recovered aqueous layer [3]. Only dimeric enzyme that was

hydrophilic remained in the supernatant. Serum, as a source of PLD, was diluted 1/100 in the MES buffer before use. The diluted serum (70 µL) was mixed with 100 µL of ALP at 200 to 300 U/L in MES buffer at pH 6.0, and incubated at 30°C for 4 h. Triton-solubilized samples had the detergent removed by butanol extraction as above, and octyl-Sepharose used to determine the amount of hydrophilic dimer. A commercial source of PLD was assessed undiluted using the same protocol as for serum.

C. Results

1. Gradient and Standard Pore Polyacrylamide Gel Electrophoresis

The majority of partially purified, high M_r ALP applied directly remained in the stacking gel in both standard pore PAGE (Plate 4.1) and in gradient gel electrophoresis (Plate 4.2). However, when solubilized with Triton X-100 or butanol, the ALP then migrated to the same position as a purified amphiphilic form of the enzyme; when the high M_r ALP was released by PI-PLC, it co-migrated with a purified hydrophilic form of the enzyme (Plate 4.1 & 4.2). The ALP in

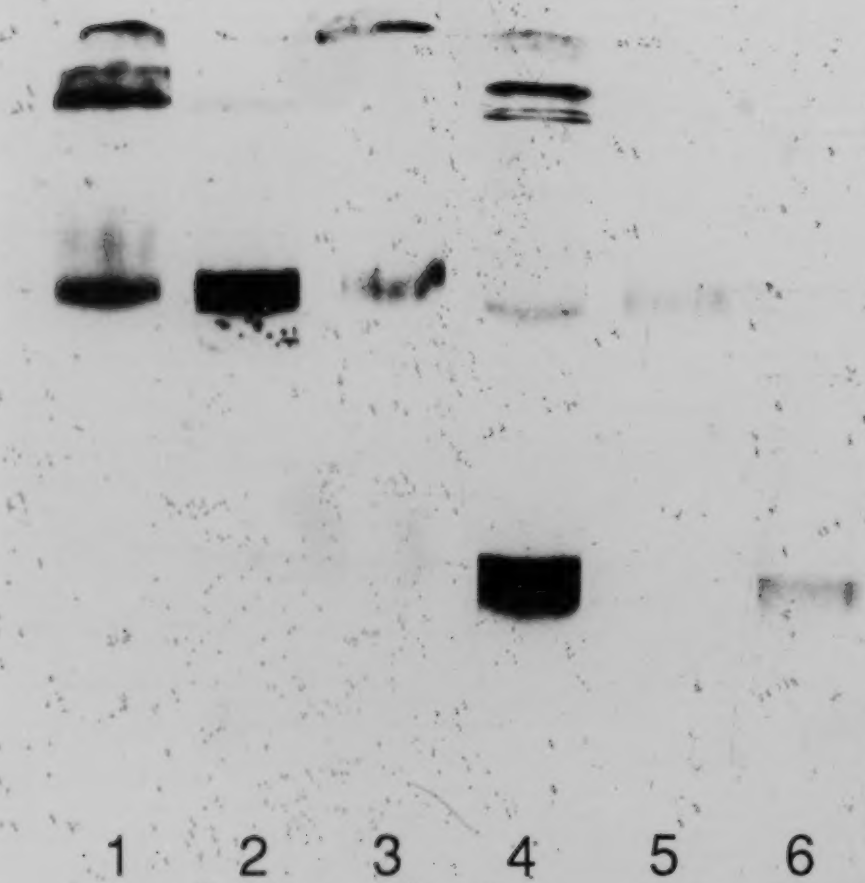

Plate 4.1 Standard pore polyacrylamide gel electrophoresis of partially purified high M_r alkaline phosphatase. Lane 1, untreated; Lane 2, solubilized with 1%(v/v) Triton X-100; Lane 3, aqueous layer after butanol treatment; Lane 4, PI-PLC released; Lane 5, purified amphiphilic ALP; Lane 6, purified hydrophilic ALP.

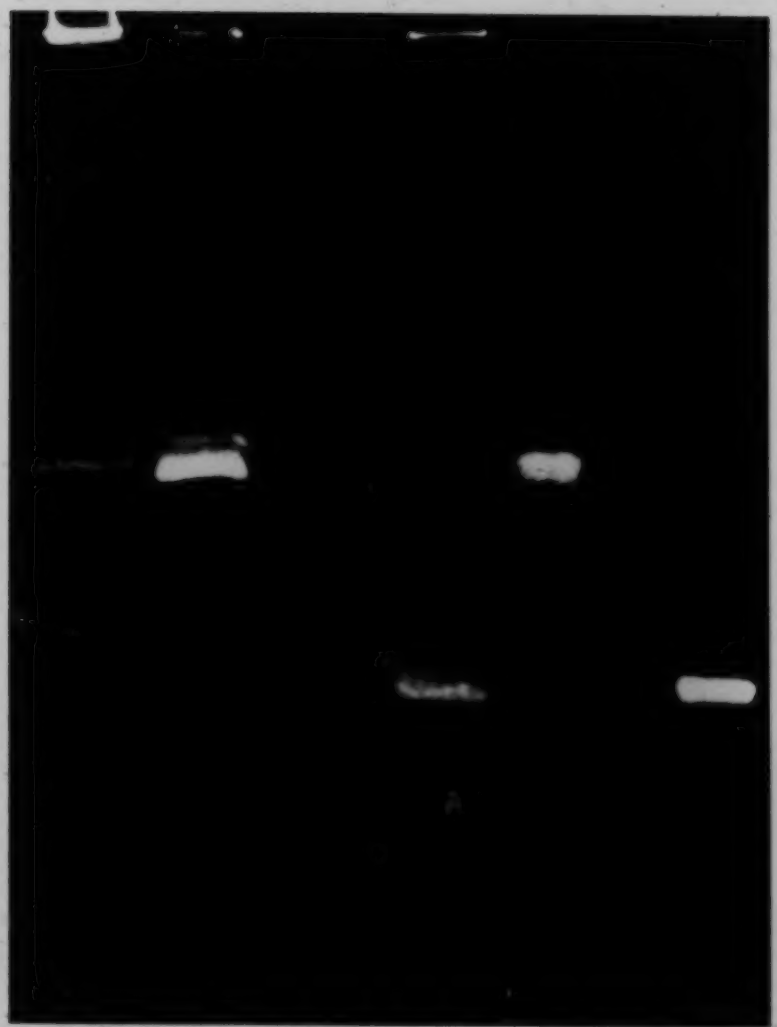

Plate 4.2 Gradient polyacrylamide gel electrophoresis of partially purified high M_r alkaline phosphatase. Lane 1, untreated; Lane 2, solubilized with 1%(v/v) Triton X-100; Lane 3, aqueous layer after butanol treatment; Lane 4, PI-PLC released; Lane 5, purified amphiphilic ALP; Lane 6, purified hydrophilic ALP.

plasma membranes behaved identically to the high M_r ALP (not shown).

2. Activation, Kinetics and Stabilities

The phospholipids and albumin had little effect on the activities of the high M_r and liver plasma membrane forms of ALP, except for some activation of high M_r ALP by phosphatidylserine and phosphatidylinositol, the anionic phospholipids (Fig. 4.1). In contrast, the soluble dimeric form of the enzyme was profoundly activated by albumin and all of the phospholipids, especially phosphatidylserine (Fig. 4.1).

The mean value for the K_m of pNPP was 3.78 mmol/L ± 0.45 (1 SD) for the three forms of ALP (high M_r, plasma membrane and hydrophilic) and was not appreciably different among them. Likewise, the tissue-specific inhibitors gave indistinguishable inhibition patterns for the three ALP forms. The percent inhibition (± 1 SD) for high M_r ALP were as follows: phenylalanylglycylglycine, 100% ± 2; phenylalanine, 96% ± 3; and homoarginine, 25% ± 2.

For hydrophilic, plasma membrane and high M_r ALP there was little difference in the heat and SDS stabilities; the mean first-order rate constant for the inactivation of the three forms of ALP by heat was 5.4×10^{-4} s^{-1} ± 0.6×10^{-4}. The mean value for inactivation by SDS was 1.3×10^{-4} s^{-1} ± 0.4×10^{-4}.

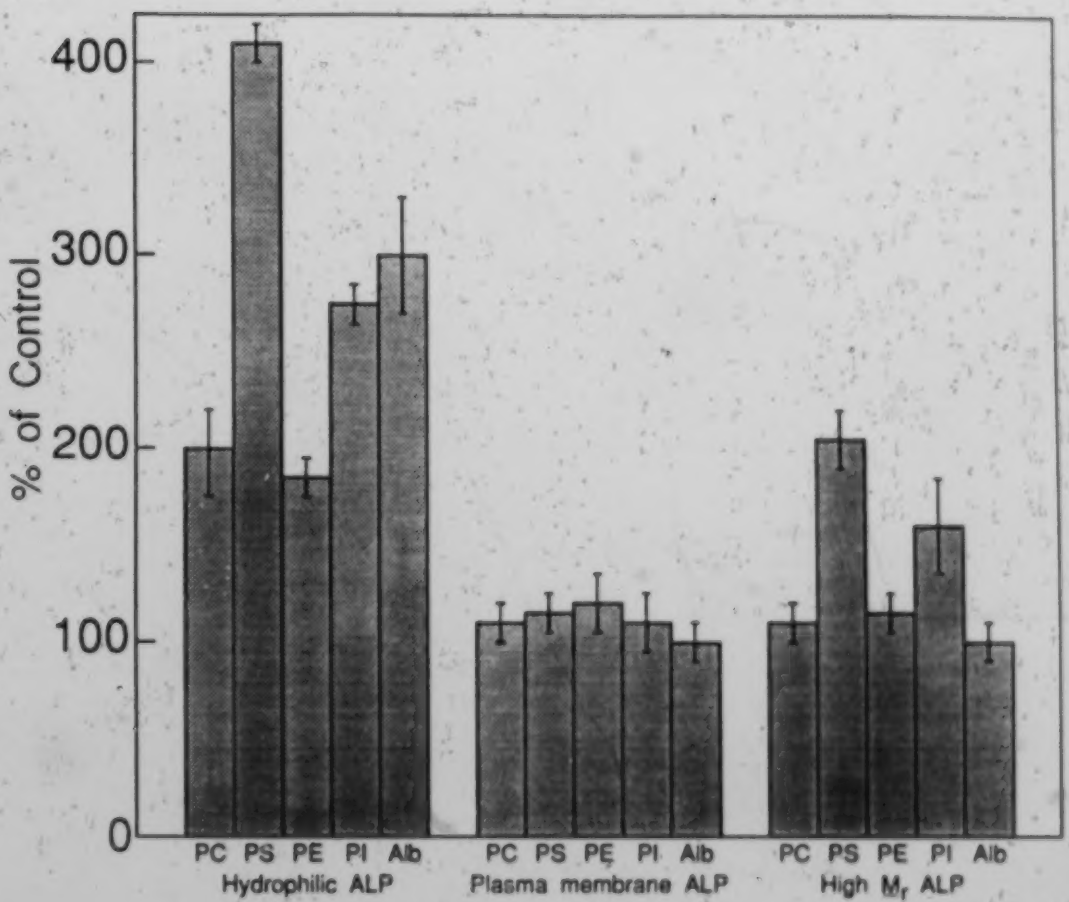

Fig. 4.1 Effect of phospholipids and albumin on activities of 3 forms of alkaline phosphatase. PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Alb, albumin. Bars represent a range of at least 3 determinations.

3. Hydrophobic and Monoclonal Antibody Interactions

The ALP solubilized by Triton X-100 or butanol from either the high M_r complex or from plasma membranes could be incorporated into liposomes and adsorbed to octyl-Sepharose, whereas the hydrophilic dimer was neither incorporated nor adsorbed (Table 4.1).

Neither high M_r ALP nor the enzyme in plasma membranes reacted with mAb. The hydrophilic dimer, on the other hand, reacted strongly (see Table 2.2).

4. Release of ALP and 5'-nucleotidase by PI-PLC

Both ALP and 5'NT were released from plasma membranes (Fig. 4.2) and from high M_r ALP that had been partially purified by gel permeation chromatography from a pool of sera (Fig. 4.3). The mean time for 50% release of ALP from its high M_r form was 10.2 h (range for 6 values, 6.6-12.0 h). For the 50% release of 5'-NT the mean time was 13.6 h (range for 3 values, 10.2-14.0). For release of ALP from plasma membranes the mean was 5.8 h (range of 20 samples, 2-16 h), and for 5'-NT the mean was 13.2 h (range of 3 values, 12.2-14.8 h). Both enzymes were also released from each of 20 different sera containing high M_r ALP, with an average percent release of 88.5% ± 9.6 (1 SD) for ALP and 87.1% ± 9.8 (1 SD) for 5'-NT. The minimum release for either enzyme in any serum was 70%.

Table 4.1 Hydrophobic Interactions of alkaline phosphatase

Enzyme Form	Incorporation into Liposomes	Adsorption to octyl-Sepharose
Purified Hydrophilic	-	-
Triton* or butanol solubilized plasma membranes	+	+
Triton* or butanol solubilized high M_r ALP	+	+

*Triton solubilizes the liposomes and interferes with adsorption to octyl-Sepharose and therefore was removed by butanol extraction before testing.

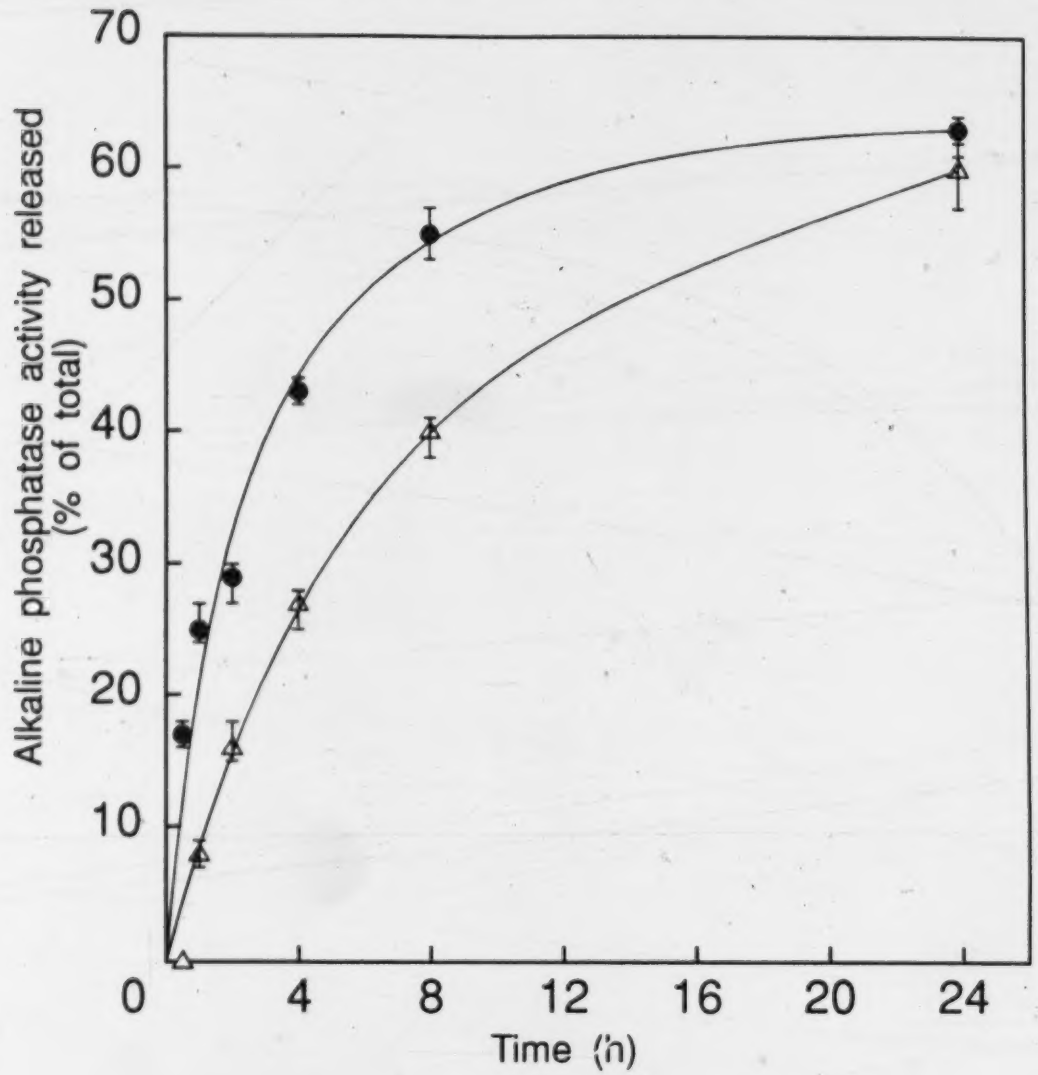

Fig. 4.2 Release of hydrophilic alkaline phosphatase (●) and 5'-nucleotidase (△) from liver plasma membranes. Enzyme forms at 300 U/L were incubated with 2 µg/mL PI-PLC at 30 °C. The amount of hydrophilic enzyme released was determined as described in the text. Bars represent a range of at least three determinations.

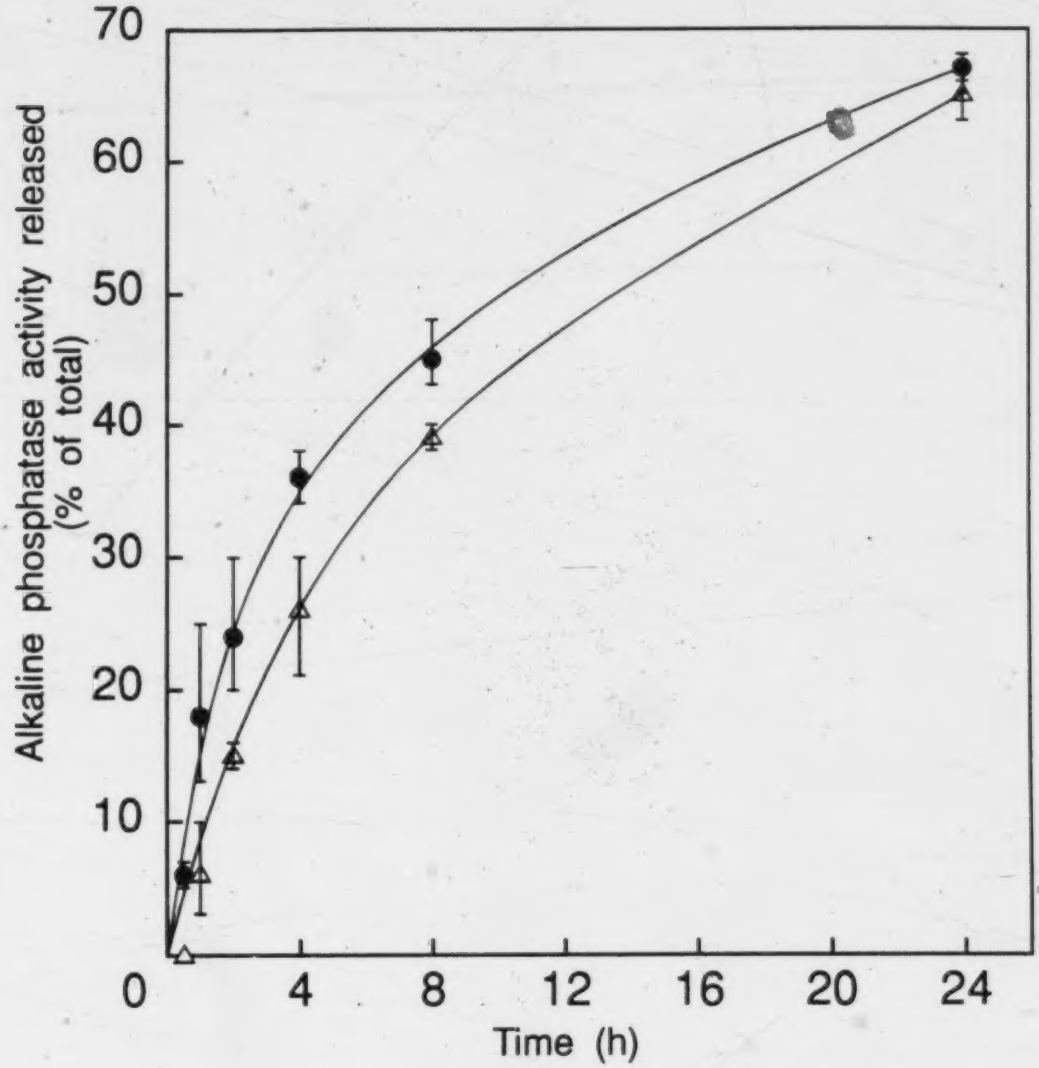

Fig. 4.3 Release of hydrophilic alkaline phosphatase (●) and 5'-nucleotidase (△) from partially purified high M_r ALP by PI-PLC. For conditions, see figure 4.2.

5. Treatment with phospholipase D enzymes

None of the phospholipase D enzymes were capable of releasing ALP from either its high M_r form or from plasma membranes (Table 4.2). Only after a Triton X-100 or butanol solubilization would the phospholipases convert the ALP to a hydrophilic form, through removal of the hydrophobic lipid tail that serves as a membrane anchor. All of the phospholipase D enzymes would, however, convert a soluble amphiphilic form of ALP to a hydrophilic form (Table 4.2).

D. Discussion

Treatments with either Triton X-100 or butanol were necessary to allow high M_r and plasma membrane forms of ALP to enter the separating gel of standard or gradient pore polyacrylamide electrophoresis (Plates 4.1 & 4.2). In both systems, the two forms of ALP migrated identically, probably as an amphiphilic species of the enzyme that still has its glycan-phosphatidylinositol membrane anchor attached [6,7,8,9,10,11,12]. In gradient pore electrophoresis solubilized ALP migrates with an apparent M_r of twice that of the hydrophilic dimer [8, Plate 4.1]. PI-PLC treatment of high M_r and plasma membrane ALP solubilized the enzyme and caused it to migrate identically to purified hydrophilic dimer that has had its hydrophobic PI anchor removed either by proteolysis or phospholipases endogenous to the

Table 4.2 Release/Conversion of Particulate and Solubilized Alkaline Phosphatase by Phospholipases

	Phospholipase D Source			
Form of ALP	Serum	Commercial	Liver	PI-PLC
Plasma membranes	-	-	-	+
Triton or butanol solubilized plasma membranes	+	+	+	+
High M_r	-	-	-	+
Triton or butanol solubilized high M_r	+	+	+	+
Purified amphiphilic	+	+	+	+

liver tissue from which it was purified. It is likely then that the PI-PLC releases ALP from both particulate forms as a hydrophilic dimer, the form of the enzyme found in normal serum [7,8,12].

The purified dimer shows considerably more activation of its catalytic activity by phospholipids and albumin than do either of the two particulate forms, which are similar in their patterns of activation (Fig. 4.1). The associated proteins and lipids of high M_r ALP and of plasma membranes may be providing the phospholipids and hydrophobic proteins needed for activation of the ALP and thus minimize activation. The purified dimer would have minimal amounts of these materials bound. Supporting this interpretation is the observation that ALP released from plasma membranes in a hydrophilic dimer form, but not separated from them, behaves as does the plasma membrane enzyme (not shown). The activation by phosphatidylserine of high M_r ALP may reflect relatively minor changes in the membrane environment of this form compared to the plasma membrane form. This may be due to select portions of the plasma membrane being shed [13,14].

No appreciable differences between the particulate forms of ALP and indeed between all forms of ALP could be found with respect to the K_m value for

p-nitrophenylphosphate, inhibition by phenylalanylglycylglycine, phenylalanine, or homoarginine, and stability to heat or SDS. These findings are not surprising in that all of the liver-type ALPs, including those from bone and kidney, are very similar in this regard [15].

ALP solubilized by Triton X-100 or butanol from high M_r particles and from plasma membranes, incorporated into synthetic liposomes and adsorbed to the hydrophobic support, octyl-Sepharose. Thus, these forms still have their hydrophobic PI anchor attached; the hydrophilic dimer does not. Also, both sources of ALP failed to incorporate or adsorb when released by PI-PLC, indicating they are hydrophilic as a result of losing their PI anchors. The electrophoretic mobilities of the products of Triton and butanol solubilization and PI-PLC release confirmed this interpretation.

The particulate forms of ALP did not react with a mAb to the enzyme. If the ALP was solubilized it then reacted in a manner indistinguishable from the purified amphiphilic form. When cleaved by PI-PLC, the forms then reacted as did the hydrophilic form. The particulate forms probably do not react with the mAb because of steric hindrance from associated protein and lipid.

The rate of release of ALP from different membranes can vary considerably. The same concentration of PI-PLC released ALP from Saos-2 cells 200X faster than from liposomes into which hydrophobic liver ALP was incorporated (Chapter 3.C.3). In the present experiments the rates of release of ALP from its high M_r form and from plasma membranes were similar, having less than a two-fold difference. There was also little difference in the rates of release of 5'-NT from the two forms. We conclude that high M_r ALP and plasma membranes have a very similar membrane composition.

The comparison of the properties of high M_r and plasma membrane forms of ALP reported herein was carried out on a pool of sera that contained the particulate enzyme form, and on a preparation of plasma membranes from human liver. It is possible that the pool of sera could include different 'compositions' of the complexed enzyme, for example association with lipoprotein-X and/or fragments of plasma membrane. The enzyme 5'-NT, like ALP, is an ecto plasma membrane enzyme and is anchored by PI in liver. It therefore should always be associated with the ALP if the particulate form of the latter is fragments of plasma membranes. This was shown to be the case in 20 randomly selected sera in which the release of both enzymes by PI-PLC was assessed. Both enzymes were released to near 100% in each serum, indicating both

are attached to the particle through their PI anchors. The most likely explanation is that the particles are fragments of plasma membranes, but a complex involving a spontaneous association of proteins, lipid and perhaps bile salts, cannot be absolutely ruled out.

The theory that lipoprotein-X forms as a result of regurgitation of bile into plasma, and then associates with plasma ALP to form a high M_r complex, is unlikely. Unassociated ALP would not have its hydrophobic membrane anchor attached and therefore would not be hydrophobic enough to allow complexation. We have shown herein that all samples of high M_r ALP examined had this anchor in place. If the enzyme were solubilized by bile salts into the circulation with its hydrophobic anchor intact, it would be cleaved by phospholipase D which is abundant in plasma [5], unless protected by lipoproteins or membrane fragments. A more plausible theory to explain the association between ALP and lipoprotein-X is that the latter, having formed in plasma as a result of the cholestatic process, interacts with fragments of either the canalicular or sinusoidal membrane that contains ALP [16].

Phospholipase D enzymes could not release ALP from either plasma membranes or high M_r forms. However, solubilization by Triton X-100 or butanol to give a soluble amphiphilic enzyme then rendered the enzyme

susceptible to cleavage by these enzymes to a hydrophilic form with removal of its membrane anchor. It could be argued that were high M_r ALP a complex with lipoprotein-X, it might, like the Triton-solubilized enzyme, be susceptible to release by phospholipase D enzymes. This did not occur with any of the sera we studied. In any case, the resistance of high M_r ALP to release by a phospholipase D from serum explains why the enzyme in the complex is not immediately cleaved off in the circulation and a reasonably stable particulate ALP results.

All of our data indicate that ALP in the high M_r complex in serum has its membrane PI anchor covalently bound; the normal soluble ALP in serum does not. Thus, high M_r ALP has not reached the circulation as a result of direct proteolytic or phospholipase cleavage; rather, the plasma membrane from which it originated must have been fragmented or partially solubilized by some mechanism. The PI hydrophobic tail of ALP must be the mechanism whereby the enzyme is attached (anchored) to the high M_r complex. It is not surprising then that many of the properties of the native and solubilized high M_r ALP are very similar to those of the enzyme in plasma membranes. We conclude that in most instances in which high M_r ALP is found in serum, it is in the form of fragments or vesicles of plasma membranes from the liver.

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Chapter 5

General Conclusions

Alkaline phosphatase from the liver and placenta use a phosphatidylinositol anchor to attach to the plasma membrane. When the enzyme is solubilized so that the anchor remains on the enzyme, it is in an amphiphilic form. The phosphatidylinositol can be removed by phosphatidylinositol phospholipase C, proteases, or endogenous phospholipases that are activated at low pH, and the enzyme becomes hydrophilic. Due to the hydrophobic anchor, the amphiphilic forms of alkaline phosphatase migrate on gradient polyacrylamide gel electrophoresis with an apparent M_r twice that of the hydrophilic form. The hydrophobic tail also increases the sensitivity of the enzyme activity to heat, the enzyme is activated less by phospholipids and albumin, and it has a greater affinity for monoclonal antibodies.

The hydrophobic phosphatidylinositol tail also allows the alkaline phosphatase to incorporate into liposomes or red cell ghosts. The amphiphilic form has the same properties whether in solution or incorporated into liposomes, so having the anchor in a membrane does not seem to affect the enzyme. Incorporation can be inhibited by hydrophobic proteins or by monoclonal antibodies. The proteins would bind to the anchor , while the antibodies must bind close enough to the anchor to cause steric hindrance. The cleavage of the anchor by phosphatidylinositol phospholipase C is considerably slower for the liposome-incorporated

enzyme than the soluble form. The rate of cleavage, and therefore the release of the alkaline phosphatase, from different membranes is quite variable. The activity of the phosphatidylinositol phospholipase C is affected greatly by the membrane environment. Release is blocked when a monoclonal antibody to the alkaline phosphatase is present. Since incorporation is also blocked by the monoclonal antibody, the binding of it must make the phosphatidylinositol unaccessible.

High M_r alkaline phosphatase seen, in the sera of patients with cholestatic liver disease, is in the amphiphilic form when solubilized by butanol or detergent. When phosphatidylinositol phospholipase C is used to release the enzyme, a hydrophilic form results. The alkaline phosphatase in this particle seems to associate as it does in the membrane. The high M_r alkaline phosphatase has kinetic, chemical and physical properties similar to those of the enzyme in liver plasma membranes. Thus, high Mr alkaline phosphatase in serum is most likely formed by fragmentation of the liver plasma membrane.

Appendix A

Compendium of Integral Membrane Proteins Anchored by Phosphatidylinositol

1. Alkaline phosphatase
   Ikezawa, H, Yamanegi,M, Taguchi, R, Miyashita, T, Ohyabu, T: Studies on phosphatidylinositol phosphodiesterase (phospholipase C type) of *Bacillus cereus*. Biochim Biophys Acta 1976;450:154-164.

   Low, MG, Finean, JB: The action of phosphatidylinositol-specific phospholipases C on membranes. Biochem J 1976;154:203-208.

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   Low, MG, Finean, JB: Specific release of plasma membrane enzymes by a phosphatidylinositol-specific phospholipase C. Biochim Biophys Acta 1978;508:565-570.

   Panagia, V, Heyliger, CE, Choy, PC, Dhalla, NS: Solubilization of rat heart sarcolemma 5'-nucleotidase by phosphatidylinositol-specific phospholipase C. Biochim Biophys Acta 1981;640:802-806.

3. Acetylcholinesterase
   Low, MG, Finean, JB: Non-lytic release of acetylcholinesterase from erythrocytes by a phosphatidylinositol phospholipase C. FEBS Lett 1977;82:143-146.

   Futerman, AH, Low, MG, Silman, I: A hydrophobic dimer of acetylcholinesterase from *Torpedo californica* electric organ is solubilized by phosphatidylinositol phospholipase C. Neurosci Lett 1983;40:85-89.

4. Thy-1 antigen
   Low, MG, Kincade, PW: Phosphatidylinositol is the membrane anchoring domain of the Thy-1 glycoprotein. Nature 1985;318:62-64.

   Tse, AGD, Barclay, AN, Watts, A, Williams, AF: A glycophospholipid tail at the carboxy terminus of the Thy-1 glycoprotein of neurons and thymocytes. Science 1985;230:1003-1008.

5. Variant surface glycoprotein (VSG) of *Trypanosomes*
   Ferguson, MAJ, Haldar, K, Cross, GAM: *Trypanosoma brucei* variant surface glycoprotein has a *sn*-1,2-dimyristyl glycerol membrane anchor at its COOH terminus. J Biol Chem 1985;260:4963-4968.

Ferguson, MAJ, Low, MG, Cross, GAM: Glycosyl-sn-1,2-dimyristylphosphatidylinositol is covalently linked to Trypanosoma brucei variant surface glycoprotein. J Biol Chem 1985;260:14547-14555.

6. Neural cell adhesion molecule (N-CAM)
He, H, Barbet, J, Chaix, J, Goridis, C: Phosphatidylinositol is involved in the membrane attachment of NCAM-120, the smallest component of the neural cell adhesion molecule. EMBO J 1986;5:2489-2494.

Sadoul, K, Meyer, A, Low, MG, Schachner, M: Release of the 120 kDa component of the mouse neural cell adhesion molecule N-CAM from cell surfaces by phosphatidylinositol-specific phospholipase C. Neurosci Lett 1986;72:341-346.

7. Protease gp63, lipophosphoglycan, glycolipids A,B,C from Leishmania
Etges, R, Bouvier, J, Bordier, C: The major surface protein of Leishmania promastigotes is a protease. J Biol Chem 1986;261:9098-9101.

Bordier, C, Etges, RJ, Ward, J, Turner, MJ, Cardoso de Almeida, ML: Leishmania and Trypanosoma surface glycoproteins have a common glycophospholipid membrane anchor, Proc Natl Acad Sci USA 1986;83:5988-5991.

Sevlever, D, Londner MV, Rosen, G: Evidence for a phosphatidylinositol anchor in glycolipid antigens of Leishmania major. Biochem Int 1989;19:59-66.

8. Decay accelerating factor (DAF)
Davitz, MA, Low, MG, Nussenzeig, V: Release of decay-accelerating factor (DAF) from the cell membrane by phosphatidylinositol-specific phospholipase C (PI-PLC). J Exp Med 1986;163:1150-1161.

Medof, ME, Walter, EI, Roberts, WL, Haas, R, Rosenberry, TL: Decay accelerating factor of complement is anchored to cells by a C-terminal glycolpid. Biochemistry 1986;25:6740-6747.

9. Trehalase
Takesue, Y, Yokota, K, Nishi, Y, Taguchi, R, Ikezawa, H: Solubilization of trehalase from rabbit renal and intestinal brush-border membranes by a phosphatidylinositol-specific phospholipase C. FEBS Lett 1986;201:5-8.

10. T cell alloantigen (RT 6.2) from Rat
Kosh, F, Theile, H, Low, MG: Phosphatidylinositol is the membrane anchoring domain of RT-6.2 and Thy-1. Transplant Proc 1987;19:3140-3143.

11. T cell activating protein (TAP)
Reiser, H, Oettgen, H, Yeh, ETH, Terhorst, C, Low, MG, Benacerraf, B, Rock, KL: Structural characterization of the TAP molecule: a phosphatidylinositol-linked glycoprotein distinct from the T cell receptor/T3 complex and Thy-1. Cell 1986;47:365-370.

12. Alkaline phosphodiesterase I
Nakabayashi, T, Ikezawa, H: Alkaline phosphodiesterase I release from eukaryotic plasma membranes by phosphatidylinositol-specific phospholipase C. I. The release from rat organs. J Biochem 1986;99:703-712.

Nakabayashi, T, Ikezawa, H: Alkaline phosphodiesterase I release from eukaryotic plasma membranes by phosphatidylinositol-specific phospholipase C. II. The release from brush border membranes of porcine intestine. Toxicon 1986;24:975-984.

13. Qa mouse T lymphocyte antigen
Stiernberg, J, Low, MG, Flaherty, L, Kincade, PW: Removal of lymphocyte surface molecules with phosphatidylinositol-specific phospholipase C: effects on mitogen responses and evidence that ThB and certain Qa antigens are membrane-anchored via phosphatidylinositol. J Immunol 1987;38:3877-3884.

14. THB mouse lymphocyte antigen
Stiernberg, J, Low, MG, Flaherty, L, Kincade, PW: Removal of lymphocyte surface molecules with phosphatidylinositol-specific phospholipase C: effects on mitogen responses and evidence that ThB and certain Qa antigens are membrane-anchored via phosphatidylinositol. J Immunol 1987;38:3877-3884.

15. Heparan sulfate proteoglycan
Ishahara, M, Fedarko, NS, Conrad, HE: Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoheparan sulfate metabolism and hepatocyte growth. J Biol Chem 1987;262;4708-4716.

16. Mouse antigen to antibody JIId
Pierres, M, Naquet, P, Barbet, J, Marchetto, S, Marics, I, Devaux, C, Barad, M, Hyman, R, Rougon, G: Evidence that murine hematopoietic cell subset marker JIId is attached to a glycosyl-phosphatidylinositol membrane anchor. Eur J Immunol 1987;17:1781-1785.

17. Mouse thymocyte proteins of 13 kDa and 52 kDa
Pierres, M, Barbet, J: Two novel phospholipid linked mouse thymocyte surface molecules released by phosphatidylinositol-specific phospholipase C. Mol Immunol 1987;24:1273-1280.

18. Temperature-specific surface antigen of Paramecium
Capdeville, Y, de Almeida, MLC, Deregnaucourt, C: The membrane anchor of Paramecium temperature-specific surface antigens is a glycosylinositol phospholipid. Biochem Biophys Res Commun 1987;147:1219-1225.

19. NAD^+-glycohydrolase
Kim, U, Rockwood, SF, Kim, H, Daynes, RA: Membrane a ssociated NAD^+ glycohydrolase from rabbit erythrocytes is solubilized by phosphatidylinositol-specific phospholipase C. Biochim Biophys Acta 1988;965:76-81.

20. Major Fc receptor in blood (FcRIII, CD16)
Selvaraj, P, Rosse, WF, Silber, R, Springer, TA: The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria. Nature 1988;333:565-567.

Huizinga, TWJ, van der Schoot, E, Jost, C, Klaasen, R, Kleijer, M, van dem Borne AEG, Roos, K, Tetteroo, PAT: The PI-linked receptor FcRIII is released on stimulation of neutrophils. Nature 1988;333:667-669.

21. Monocyte differentiation antigen (CD14)
Haziot, A, Chen ,S, Ferrero, E, Low, MG, Silber, R, Goyert, SM: The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. J Immunol 1988;141:547-552.

22. Lymphocyte associated antigen 3 (LFA-3)
Selvaraj, P, Dustin, ML, Silber, R, Low, MG, Springer, TA: Deficiency of lymphocyte function-associated antigen 3 (LFA-3) in paroxysmal nocturnal hemoglobinuria. Functional correlates and evidence for a phosphatidylinositol membrane anchor. J Exp Med 1987;166:1011-1025.

23. Lipoprotein lipase
Chan, BL, Lisanti, MP, Rodriguez-Boulan, E, Saltiel, AR: Insulin stimulated release of lipoprotein lipase by metabolism of its phosphatidylinositol anchor. Science 1988;241:1670-1672.

24. Peanut agglutinin-binding glycoprotein (oligodendrocyte-myelin protein)
Mikol. DD, Stefansson, K: A phosphatidylinositol-linked peanut agglutinin-binding glycoprotein in central nervous system myelin and on oligodendrocytes. J Cell Biol 1988;106:1273-1279.

25. Renal dipeptidase
Hooper, NM, Low, MG, Turner, AJ: Renal dipeptidase is one of the membrane proteins released by phosphatidylinositol-specific phospholipase C. Biochem J 1987;244:465-469.

26. Arylacylamidase
Majumdar, R, Balasubramanian, AS: Essential and non-essential phosphatidylinositol residues in acetylcholinesterase and arylacylamidase of sheep basil ganglia. FEBS lett 1982;146:335-338.

27. Ly-6 A,C, and E antigens
Hammelburger, JW, Palfree, RGE, Sirlin, S, Hammerling, U: Demonstration of phosphatidylinositol anchors on Ly-6 molecules by specific phospholipase digestion and gel electrophoresis in octylglucoside. Biochem Biophys Res Commun 1987;148:1304-1311.

28. 130 kDA major glycoprotein from rat hepatoma
Ikehara, Y, Hayashi, Y, Ogata, S, Miki, A, Kominami, T: Purification and characterization of a major glycoprotein in rat hepatoma plasma membranes. One of the major proteins released by phosphatidylinositol-specific phospholipase C. Biochem J 1987;241:63-70.

29. Scrapie prion protein
Stahl, N, Borchelt, DR, Hsiao, K, Prusiner, SB: Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell 1987;51:229-240.

30. 46, 48, 158 kDa proteins from PC12 pheochromocytoma cells, and at least 8 PI-anchored proteins in brain
Margolis, RK, Goossen ,B, Margolis, U: Phosphatidylinositol-anchored glycoproteins of the PC12 pheochromocytoma cells and brain. Biochemistry 1988;27:3454-3458.

31. Carcinoembryonic antigen (CEA)
Jean, F, Malapert, P, Rougon, G, Barbet, J: Cell membrane, but not circulating, carcinoembryonic antigen is linked to a phosphatidylinositol-containing hydrophobic domain. Biochem Biophys Res Commun 1988;155:794-800.

32. OX-45 from rat
Robinson ,PJ, Spencer, SC: Phosphatidylinositol-anchored membrane proteins and lymphocyte activation. Immunol Lett 1988;19:85-94.

33. p76 merozoite protease from Plasmodium falciparum
Braun-Breton, C, Pereira da Silva, L: Activation of a Plasmodium falciparum protease correlated with merozoite

maturation and erythrocyte invasion. Biol Cell 1988;64:223-231.
Braun-Breton, C, Rosenberry, TL, Pereira da Silva, L: Induction of the proteolytic activity of a membrane protein in Plasmodium falciparum by phosphatidylinositol-specific phospholipase C. Nature 1988;332:457-459.

34. Blast-1 from human lymphocytes
Kuroki, M, Arakawa, F, Yamamoto, H, Shimura, H, Ikehara, Y, Matsuoka, Y: Active production and membrane anchoring of carcinoembryonic antigen observed in normal colon mucosa. Cancer Lett 1988;43:151-157.

35. Contact site A in Dictyostelium discoideum
Stadler, J, Keenan, TW, Bauer, G, Gerisch, G: The contact site A glycoprotein of Dictyostelium discoideum carries a phospholipid anchor of a novel type. EMBO J 1989;8:371-377.

36. Ssp-4 in Trypanosoma cruzi
Andrews, NW, Robbins, ES, Ley, V, Hong, KS, Nussenzweig, V: Developmentally regulated, phospholipase C-mediated release of the major surface glycoprotein of amastigotes of Trypanosoma cruzi. J Exp Med 1988;167:300-314.

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Roy-Choudhury, S, Mishra, VS, Low, MG, Das, M: A phospholipid is the membrane-anchoring domain of a protein growth factor of molecular mass 34 kDa in placental trophoblasts. Proc Natl Acad Sci USA 1988;85:2014-2018.

38. 3 major surface antigens in Schistosoma mansoni
Low, MG, Saltiel, AR: Structural and functional roles of glycosyl-phosphatidylinositol in membranes. Science 1988;239:268-275.

Pearce, EJ, Sher, A: Three major surface antigens of Schistosoma mansoni are linked to the membrane by glycosylphosphatidylinositol. J Immunol 1989;142:979-984.

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Phelps, BM, Primakoff, P, Koppel, DE, Low, MG, Myles, DG: Restricted lateral diffusion of PH-20, a PI-anchored sperm membrane protein. Science 1988;240:1780-1782.

40. GP-2 from pancreatic zymogen granule
LeBel, D, Beattie, M: The major protein of pancreatic zymogen granule membranes (GP-2) is anchored via covalent bonds to phosphatidylinositol. Biochem Biophys Res Commun 1988;154:818-823.

41. 140 kDa protein from cultured astrocytes
Amblard, F, He, J-T, Barbet, J, Goridid, C, Prochiantz, A: A 140-kilodalton protein is released from cultured astrocytes by phosphatidylinositol phospholipase C. J Neurochem 1988;50:486-489.

42. Transferrin receptor
Haldar, K, Henderson, CL, Cross, GAM: Identification of the parasite transferrin receptor of Plasmodium falciparum-infected erythrocytes and its regulation via 1,2-diacyl-sn-glycerol. Proc Natl Acad Sci USA 1986;83:8565-8569.

43. TN-368 ß-glucosidase from moth ovary
Ikezawa, H, Hashimoto, A, Taguchi, R, Nakabayashi, T, Himeno, M: Release of PI-anchoring enzymes and other effects of phosphatidylinositol-specific phospholipase C from Bacillus thuringiensis on TN-368 cells from a moth ovary. Toxicon 1989;27:637-645.

44. Folate binding protein
Lacey, SW, Sanders, JM, Rothberg, KG, Anderson, RGW, Kamen, BA: Complementary DNA for the folate binding protein correctly predicts anchoring to the membrane by glycosyl-phosphatidylinositol. J Clin Invest 1989;84:715-720.

45. Elongation factor (EF-1α)
Hayashi, Y, Urade, R, Utsami, S, Kito, M: Anchoring of peptide elongation factor EF-1 by phosphatidylinositol at the endoplasmic reticulum membrane. J Biochem 1989;106:560-563.

46. CD-59 (membrane inhibitor of reactive lysis, MIRL, IF5 Antigen)
Taguchi, R, Funahashi, Y, Ikezawa, H, Nakashima, I: Analysis of PI(phosphatidylinositol)-anchoring antigens in a patient of paroxysmal nocturnal hemoglobinuria (PNH) reveals deficiency of IF5 antigen (CD59), a new complement-regulatory factor. FEBS Lett 1990;261:142-146.

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Hansch GM, Hammer, CH, Vanguri, P, Shin, ML: Homologous species restriction in lysis of erythrocytes by terminal complement proteins. Proc Natl Acad Sci USA 1981;78:5118-5121.

Hansch, GM, Weller, PF, Nicholson-Weller, A: Release of C8 binding protein (C8bp) from the cell membrane by phosphatidylinositol-specific phospholipase C. Blood 1988;72:1089-1092.

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Stefanova, I, Hilgert, I, Kristofova, H, Brown, R, Low, MG, Horejsi, V: Characterization of a broadly expressed human leukocyte surface antigen MEM-43 anchored in membrane through phosphatidylinositol. Mol Immunol 1989;26:153-161.

49. FMLP receptor on neutrophils
Skubitz, KM, Lakatua, JDA: Identification of the major glycosyl-phosphatidylinositol anchored proteins on the surface of human neutrophils. J Leuk Biol 1990;48:50-59.

50. 26 kDa maturation glycoprotein from rat epididymal sperm
Moore, A, White, TW, Ensrud, KW, Hamilton, DW: The major maturation glycoprotein found on rat cauda epididymal sperm surface is linked to the membrane via phosphatidylinositol. Biochem Biophys Res Commun 1989;160:460-468.

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Shamamori, Y, Kumagai, Y, Watanabe, Y, Fugimoto, Y: Human placental carboxypeptidase M is anchored by a glycosyl-phosphatidylinositol moiety. Biochem Int 1990;20:607-613.

52. Lipoarabinomannan, lipomannan of Mycobacterium tuberculosis
Hunter, SW, Brennan, PJ: Evidence for the presence of a phosphatidylinositol anchor on the lipoarabinomannan and lipomannan of Mycobacterium tuberculosis. J Biol Chem 1990;265:9272-9279.

53. Kidney microvillar dipeptidase
Hooper, NM, Low, MG, Turner, AJ: Renal dipeptidase is one of the membrane proteins released by phosphatidylinositol-specific phospholipase C. Biochem J 1987;244:465-469.

Hooper, NM, Keen, JN, Turner, AJ: Characterizaton of the glycosyl-phosphatidylinositol-anchored human renal dipeptidase reveals that it is more extensively glycosylated than the pig enzyme. Biochem J 1990;265:429-433.

54. Kidney microvillar aminopeptidase P
Hooper, NM, Turner, AJ: Ectoenzymes of the kidney microvillar membrane. FEBS Lett 1988;229:340-344.

55. Thy-3
Naquet, P, Barbet, J, Pont, S, Marchetto, S, Barad, M, Deveaux, C, Rougon, G, Pierres, M:Characterization of Thy-1 with monoclonal antibodies and evidence of Thy-3.(1989) in Cell surface antigen Thy-1:Immunology, neurology and therapeutic applications (Reif, AE, Schlesinger, M, eds),99-117, Marcel Dekker, New York.

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Hinoda, Y, Neumaier, M, Hefta, SA, Drzeniek, Z, Wagener, C, Shively, L, Hefta, LJF, Shively, JE, Paxton, RJ: Molecular cloning of a cDNA coding biliary glycoprotein 1: Primary structure of a glycoprotein immunologically crossreactive with carcinoembryonic antigen. Proc Natl Acad Sci USA 1988;85:6959-6963.

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Mizukami, SD, Vinjamuri, SD, Todd, RF: The Mo3 activation antigen of human mononuclear phagocytes is a phosphatidylinositol(PI)-linked membrane glycoprotein. FASEB J 1989;3:A963-A963.

58. CD48
Low, MG: The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim Biophys Acta 1989;988:427-454.

59. 90 kDa glycoprotein from Trypanosoma cruzi
Low, MG: The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim Biophys Acta 1989;988:427-454.

60. Procyclin from Trypanosoma brucei
Clayton, CE, Mowatt, MR: The procyclic acidic repetitive proteins of Trypanosome brucei. J Biol Chem 1989;264:15088-15093.

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Haldar, K, Ferguson, MAJ, Cross, GAM: Acylation of a Plasmodium falciparum merozoite surface antigen via sn-1,2-diacyl glycerol. J Biol Bhem 1985;260:4969-4974.

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Nagel, SD, Boothroyd, JC: The major surface antigen, P30, of Toxoplasma gondii is anchored by a glycolipid. J Biol Chem 1989;264:5569-5574.

63. F-11, a 130 kDa chick neural cell surface glycoprotein
Brummendorf, T, Wolff, JM, Frank, R, Rathjen, FG: Neural recognition molecule F11: homology with fibronectin type III and immunoglobulin type C domains. Neuron 1989;2:1351-1361.

Wolff, JM, Brummendorf, T, Rathjen, FG: Neural cell recognition molecule F11: membrane interaction by covalently attached phosphatidylinositol. Biochem Biophys Res Commun 1989;161:931-938.

64. Antigen 117 from Dictyostelium discoideum
Sadhegi, H, Williams, K, Klein, C: Characterization of antigen 117. J Biol Chem 1987;262:16294-16299.

65. 125 kDa glycoprotein from Saccaromyces cerevisiae (GAS1)
Conzelmann, A, Riezman, H, Desponds,C, Bron, C: The major 125 kDa membrane glycoprotein of Saccaromyces cerevisiae is attached to the lipid bilayer through an inositol-containing phospholipid. EMBO J 1988;7:2233-2240.

Nuoffer, C, Jeno, P, Conzelmann, A, Riezman, H: Determinants for glycophospholipid anchoring of the Saccaromyces cerevisiae GAS1 protein to the plasma membrane. Mol Cell Biol 1991;11:27-37.

66. Sgp-1, Sgp-2 of squid neural tissue
Low, MG: The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim Biophys Acta 1989;988:427-454.

67. 150 kDa glycoprotein from human platelets
Dhar, A, Shukla, SD: Release of human platelet surface glycoprotein by phosphatidylinositol-specific phospholipase(s) C. J Cell Biol 1988;107:559a-559a.

68. 82 and 68 kDa proteins from bovine adrenal chromaffin granule
Fouchier, F, Bastiani, P, Baltz, T, Aunis, D, Rougon, G: Glycosylphosphatidylinositol is involved in the membrane attachment of proteins in granules of chromaffin cells. Biochem J 1988;256:103-108.

69. I-antigenic glycoprotein GP-3
Edge, ASB ,Spiro, RG: Thyroid cell surface glycoproteins. J Biol Chem 1985;260:15332-15338.

70. H-2L^d hybrid
Mann, DW, Stroynowski, I, Hood, L, Forman, J: An H-2L^d hybrid molecule with a QA-2 3 domain and phosphatidylinositol anchor is not recognized by H-2L^d-specific cytotoxic T lymphocytes. J Immunol 1989;142:318-322.

71. Mouse Ly-31.1
Immunogenetics 1989;29:235-240.

72. Choline-O-acetyltransferase
Carnoll, PT, Smith, LK: Effect of phospholipase C from Bacillus cereus on the release of membrane-bound cholin-O-acetyltransferase from rat hippocampal tissue. J Neurochem 1990;54:1047-1055.

73. Uromodulin (Tamm-Horsfall glycoprotein, uromucoid)
Rindler, MJ, Naik, SS, Li, N, Hoops, TC: Uromodulin (Tamm Horsfall glycoprotein/uromucoid) is a phosphatidylinositol-linked membrane protein. J Biol Chem 1990;265:20784-20789.

74. Cellular receptor for urokinase plasminogen activator
Ploug, M, Ronne, E, Behrendt, N, Jensen, AL, Blasi, F, Dano, K: Cellular receptor for urokinase plasminogen activator. Carboxy-terminal processing and membrane anchoring by glycosyl-phosphatidylinositol. J Biol Chem 1991;266:1926-1933.

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Skubitz, KM, Stroncek ,DF, Sun, B: Neutrophil-specific antigen NB1 is anchored via a glycosyl-phosphatidylinositol linkage. J Leuk Biol 1991;49:163-171.

Appendix B

Publications arising from this work

1. Hawrylak, K, Kihn, L, Rutkowski, D, Stinson, RA: Purified tetrameric alkaline phosphatase: the effect of treatments with phosphatidylinositol phospholipase C and sodium dodecyl sulfate. Clin Chim Acta 1990;186:197-202.

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3. Kihn L, Dinwoodie A, Stinson RA: High molecular weight alkaline phosphatase in serum has properties similar to the enzyme in plasma membranes of liver. Am J Clin Pathol, in press.

4. Kihn L, Rutkowski D, Nakatsui D, Stinson RA: Properties of amphiphilic and hydrophilic forms of alkaline phosphatase from human liver. Enzyme, in press.